ANALYSIS OF THE CELLULAR AND MOLECULAR MECHANISMS WHICH UNDERLIE SENSITIVITY TO BACTERIAL ENDOTOXIN AND EARLY TOLERANCE

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ABSTRACT

Title of Dissertation: Analysis of the Cellular and Molecular Mechanisms Which Underlie Sensitivity to Bacterial Endotoxin and Early Endotoxin Tolerance

Beth E. Henricson, Doctor of Philosophy, 1992.

Dissertation directed by: Stefanie N. Vogel, Professor, Dept. of Microbiology

Gram negative sepsis is increasing in frequency at present due to an increased number of patients at risk. Chemotherapy, AIDS related immune suppression, and complicated surgical interventions predispose already compromised patients to intractable infection. Gram negative bacterial endotoxin (lipopolysaccharide or LPS) is a causative factor in septic shock. LPS can elicit both toxic and benefical effects, which have been suggested to be cytokine-mediated. The phenomenon of "early endotoxin tolerance," which is induced by sublethal exposure to LPS, results in a transient period of hyporesponsiveness that is most profound at three to four days after exposure, and is marked by reduced cytokine production after a challenge exposure to LPS. Early endotoxin tolerance is also inducible by the non-toxic LPS derivative monophosphoryl lipid A, although a larger dose is required to induce a level of tolerance equivalent to that induced by LPS. Equivalent tolerance-inducing doses of LPS and MPL were compared for their ability to induce several cytokines. Although LPS- and MPL-induced colony stimulating factor (CSF) activity was comparable for doses of LPS and MPL that elicited an equivalent

state of early endotoxin tolerance, levels of tumor necrosis factor (TNF), Interleukin-6, Interleukin-1, and interferon were significantly lower in MPL-injected mice. These results suggest that the lowered toxicity of MPL may be related to its elicitation of significantly lower levels of potentially toxic intermediaries. Administration of a recombinant Interleukin-1 receptor antagonist protein to mice was found to inhibit induction of colony stimulating factor, as well as induction of "early endotoxin tolerance," by LPS . LPS-induced hypoglycemia was also significantly reversed by recombinant Interleukin-1 receptor antagonist. These findings provide direct evidence that Interleukin-1 and Tumor Necrosis Factor are intermediates in these lipopolysaccharide-induced phenomena. When the interaction of LPS and MPL with cells was compared by electron microscopy and studies in which LPS and MPL were compared for their capacities to displace a monospecific anti-LPS receptor antibody, it appeared that LPS interacted with cells more strongly than MPL. Furthermore, LPS- and MPL-induced effects, such as TNF secretion and induction of *in vitro* tolerance in macrophages, were differentially blocked by the novel LPS antagonist *Rhodopseudomonas sphaeroides* DPLA, suggesting that the two species interact with macrophages with differing binding capacities. When the expression of several LPS-inducible macrophage "early genes" was used as an additional approach for assessing LPS-responsiveness in macrophages, LPS was found to induce all seven genes at a significantly lower concentration than MPL by approximately 100-1,000 fold, and the genes may be catagorized into groups by comparison of patterns of LPS- *versus* MPL-induced mRNA and the expression of the same genes after induction of tolerance *in vitro*. Taken collectively, these findings suggest that the differential bioactivities induced by LPS and other toxic derivatives *versus* non-toxic MPL, may in large part, be attributed to a less avid interaction of MPL with macrophages.

ANALYSIS OF THE

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by

Beth E. Henricson

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The pursuit of the degree of Doctor of Philosophy at my stage of life has been, quite literally, a life threatening experience. It was not a venture that I embarked upon as a lark, nor because I was bored with my life. Rather, I started out five years ago to complete what I considered to be an unfinished part of my development; to complete to my satisfaction the actualization of whatever potential I had from birth; to observe; to question; to understand; to become formally what I know I have always been...a scientist.

"Some people look at the way things are and ask, "Why?" I dream of the way things could be and say, "Why not?""

-Robert F. Kennedy

For this chance to dream my own view of the future, I thank Stefanie Vogel, who leads by example, not abuse, and who succeeds by ensuring the success of others, and by creating opportunities for students to learn and grow. She routinely reaches back to help others along the path she has struggled to make. Friends and family have encouraged me and helped me to realize my goal, and I would like to acknowledge their guidance, support, and friendship. Thank you all! And so, here you are: all the people to whom I owe so much.

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Erik, my son, who calls me "Dr. Mom, woman with a mission!" Raising you was an adventure and a pleasure. I love and respect the man you've become.

Karen, my daughter, who is never without a song, and who can bring Mozart to life. Thank you for your song and your determination; it gives me strength.

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Invictus Maneo

To Larry

Virtus Sola Nobilitat

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ABBREVIATIONS

AOAH- Acyloxyacyl hydrolase enzyme

bp- base pairs

BSA- bovine serum albumin

cAMP- cyclic adenosine 5'-monophosphate

cDNA- complementary deoxyribonucleic acid

CO_2- carbon dioxide

CsCl- cesium chloride

CSF- Colony Stimulating Factor

dCTP- deoxycytidine 5'-triphosphate

DAG- diacylglycerol

DPLA- diphosphoryl lipid A

ELISA- Enzyme-Linked Immunosorbant Assay

FCS- fetal calf serum

^{3}H-LPS- tritiated lipopolysaccharide

HEPES- N-2-hydroxy-ethyl piperazine-N'-2-ethanesulfonic acid

GAF- Glucocorticoid Antagonizing Factor

IFN- Interferon

IFN-α/β- Interferon-alpha/ beta

IFN γ- Interferon gamma

IgG- Immunoglobulin G

IL- Interleukin

IP-10- Interferon-γ-inducible protein 10

I.U.- International Units

kDa- kilodalton

KDO- 2-keto-3-deoxy-D-mannooctulosonate

LD_{50}- 50% lethal dose

LPS- lipopolysaccharide

Lps- lipopolysaccharide response gene

Lps^d- *Lps* gene, defective allele

Lps^n- *Lps* gene, normal allele

M- Molar

mM- millimolar

mRNA- messenger ribonucleic acid

µg- micrograms

µl- microliters

ml- milliliters

MPL- Monophosphoryl Lipid A

NaCl- sodium chloride

ng- nanograms

nm- nanometers

n.u.- neutralizing units

OsO_4- osmium tetroxide

PAF- Platelet Activating Factor

PAGE- polyacrylamide gel electrophoresis

PBS- phosphate buffered saline

pH- log hydronium ion concentration

PEPCK- phosphoenolpyruvate carboxykinase

pg- picograms

PMSF- phenylmethylsulfonyl fluoride

rIL-1ra- recombinant Interleukin 1 receptor antagonist

ReLPS- Re rough chemotype lipopolysaccharide

RES- reticuloendothelial system

RNA- ribonucleic acid

rpm- revolutions per minute

RsDPLA- *Rhodopseudomonas sphaeroides* diphosphoryl lipid A

RsMPLA- *Rhodopseudomonas sphaeroides* monophosphoryl lipid A

SDS- sodium dodecyl sulfate

SRBC- sheep red blood cells

SSC- sodium chloride-sodium citrate solution

TNF- Tumor Necrosis Factor

V- Volts

vol- volume

Introduction

Endotoxic shock: An Ancient Disease; a Modern Menace

In recent years in the United States, an estimated 425,000 people yearly were afflicted with sepsis as a life-threatening complication of illness or injury (CDC, 1990). Fully 170,000 of those indivduals suffered from sepsis in which the invading bacterium was a Gram negative organism and > 70,000 succumbed as a result of septic shock, an increase of 139% in just eight years (CDC, 1990). At presentation, patients in Gram negative septic shock, by definition, suffer from Gram negative bacteremia and two or more of the following symptoms: severe hypotension, fever, hypoglycemia, diarrhea/weight loss, tachycardia, tachypnea, hypertriglyceridemia, thrombocytopenia, metabolic acidosis, acute renal failure, hepatic failure, acute respiratory distress syndrome, disseminated intravascular coagulation, and distributive shock (Gorelick, 1991). The number of people at risk to develop Gram negative sepsis has increased drastically within the last few years because of increased numbers of people undergoing complicated invasive surgical procedures, increased numbers of patients immunocompromised by AIDS, and higher numbers of cancer patients that are chronically immunocompromised by chemotherapy and are therefore predisposed to develop severe life-threatening infection (CDC, 1990).

Historically, sepsis was first demonstrated by John Hunter, a Scottish physician, in 1774. In 1856, German pathologist Rudolph Virchow gave what was to become the classic description of septicemia (Schmidt, 1959), although the association of symptoms such as fever with infection has been handed down

since the time of Hippocrates (reviewed in Kluger, 1979). The chief reason that Gram negative septic shock was and remains so deadly is that Gram negative bacteria contain in their outer membranes an inflammatory and immunomodulatory substance that Pfeiffer, in 1892, designated "endotoxin" to distinguish it from classic bacterial exotoxins (reviewed by Martin, 1991). In 1909, 39 cases of *Bacterium coli* (now *Escherichia coli*) sepsis were reviewed by the German physician, Jacob (Jacob, 1909). The symptomatology associated with endotoxemia was first noted in modern times in 1928 by Schwartzman, who first described the systemic disseminated intravascular coagulation and localized hemorrhagic necrosis of the syndrome that bears his name (reviewed in Martin, 1991; Abbas *et al.*, 1991). Endotoxin was reported to be the cause of shock, organ injury, and death in association with Gram negative bacteremia by Franke, in 1944 (Martin, 1991).

Endotoxin is released from the outer membranes of dead and dying bacteria during infection, and is increased in the circulation despite timely and appropriate antibacterial therapy (Bryan *et al.*, 1983; Rokke *et al.*, 1988). It is composed of bacterial lipopolysaccharide (LPS) and associated proteins from the outer bacterial membrane (reviewed in Burrell, 1990), and although the associated proteins also have a stimulatory activity of their own (Hogan and Vogel, 1987;1988), lipid A, the most toxic fraction of LPS, has been called the "endotoxic center" of endotoxin's biologic effects (Reitschel *et al.*, 1987; 1980). It is LPS that is responsible for a plethora of toxic symptoms so severe that there is an average 25% mortality rate for shock victims (CDC, 1990).

Cytokines as Mediators of Endotoxin Effects

During the 1970's and 1980's, many LPS-induced cytokine molecules were isolated and purified, and subsequently cloned and expressed (reviewed in

Vogel and Hogan, 1990; Manthey and Vogel, 1992; Abbas *et al.*, 1991). The fact that LPS-induced cytokines are the direct mediators of the pathophysiology associated with septic shock was demonstrated by: (i) induction of LPS-like symptoms following injection of the purified recombinant cytokines, and (ii) reversal of LPS-induced toxicity by administration of specific cytokine antagonists (reviewed in Vogel, 1992). It is now known that a single exposure to lipopolysaccharide elicits a "temporal hierarchy" in the appearance of cytokine molecules that act on remote target tissues (reviewed in Vogel and Hogan, 1990). Among the cytokines found to be important in response to endotoxin are those called "early acute phase reactants" that arise in the circulation between one and six hours after LPS exposure (Griesman and Woodward, 1965; Sipe and Rosenstreich, 1981; Sipe, 1990), such as tumor necrosis factor (TNF; Beutler *et al.*, 1985, Beutler and Cerami, 1987, 1989; Dinarello *et al.*, 1986), interleukin 1 (IL-1; Kampschmidt *et al.*, 1973; reviewed in Oppenheim *et al.*, 1991), interferons (IFN; Youngner and Stinebring, 1965; Youngner and Feingold, 1967; Ho *et al.*, 1967;1970; reviewed in Friedman, 1990), glucocorticoid antagonizing factor (GAF; Moore *et al.*, 1978; Goodrum and Berry, 1979), interleukin 6 (IL-6; Van Snick *et al.* 1986; Aarden, 1987), interleukin 8 (IL-8; Oppenheim *et al.*, 1991; Van Zee *et al.*, 1991; Porat *et al.*, 1992), and colony stimulating factors (CSF; reviewed by Pluznik, 1983; Williams *et al.*, 1983). In particular, Interleukin 1 (IL-1), which was discovered as an "endogenous pyrogen" (Atkins and Wood, 1955; Atkins, 1960), has been associated with the production of fever via a prostaglandin-mediated increase in the temperature set point of the hypothalamus (Snell and Atkins, 1968), as well as a multitude of proinflammatory effects including an increase in lipolysis, increases in osteoclast and collagenase activity, increases in vascular endothelial and smooth muscle proliferation, and stimulation of the adrenals to produce glucocorticoids (reviewed in Oppenheim *et al.*, 1991; Salkowski and

Vogel, 1992). TNF, which is also pyrogenic, has been shown to induce two peaks of fever in rabbits that are both prostaglandin-mediated. The first peak is induced by TNF and the second due to TNF-induced IL-1 (Dinarello *et al.*, 1986). TNF has been shown to have proinflammatory effects as diverse as IL-1, since it stimulates neutrophil adhesion to vascular endothelial cells, induces synthesis of CSFs, and activates inflammatory lymphocyte killing of bacteria (reviewed in Abbas *et al.*, 1991). TNF also mediates hypotension by synergizing with platelet activating factor to increase vascular permeability, allowing extravasation of fluid into the tissues and hemoconcentration (Floch *et al.*, 1989; Rabinovici *et al.*, 1990, 1991). Both TNF and IL-1 have been found to mediate LPS-induced hypoglycemia (Bauss *et al.*, 1987; Del Rey and Besedofsky, 1987; Vogel *et al.*, 1990). IL-1 and TNF have been found to synergize for the production of a number of toxic effects and have each been implicated in the induction by hepatocytes of a group of plasma proteins that collectively are called "late phase reactants" (Sipe and Rosenstreich, 1981). These "late phase reactants" are elevated maximally 18 to 24 hours after exposure to LPS. They consist of plasma proteins such as: fibrinogen, which has been implicated in the pathology associated with deseminated intravascular coagulation; C reactive protein, which is a known bacterial opsonin; and serum amyloid A (Sipe, 1990). In addition, several other proteins with the ability to bind LPS, such as lipid A binding protein (LBP), are induced as "late phase reactants" and LBP is felt to contribute to the clearance of LPS from the circulation (Tobias *et al.*, 1985; 1988; 1989; Schumann *et al.*, 1990). More recently, a role for IL-6 in the potentiation of IL-1- and TNF-mediated responses has also been established (Neta *et al.*, 1992).

Although administration of LPS can certainly result in toxicity, appropriate timing and dosage can actually result in many beneficial consequences which may be capitalized upon for new clinical therapeutic

interventions. Exposure to LPS increases non-specific resistance to infection by other organisms (Rowley, 1955; reviewed in Vogel and Hogan, 1990). It serves as an excellent adjuvant in enhancement of antigenic responses, including responses to antigens that are imperfectly recognized by the immune system, such as pneumococcal polysaccharide (Hiernaux *et al.*, 1989) or those that are presented by malignant tumors (O'Malley *et al.*, 1963). LPS also stimulates protection of animals from lethal irradiation that is in part mediated by IL-1 and TNF (Hanks and Ainsworth, 1965; Neta *et al.*, 1986; 1992). One effect of endotoxin exposure which is generally thought to be beneficial is the induction of "early phase endotoxin tolerance" (see below) which is a transient state of hyporesponsiveness to LPS that is produced experimentally in response to a single sublethal exposure to endotoxin. But, endotoxin tolerance has more recently been implicated in serious hyporesponsiveness to chronic infection (Peterson, 1983; 1985). Although it is protective in the short term from the devastating effects of endotoxic shock, the true contribution of tolerance to mortality via immune suppression during chronic infection or in already immune suppressed patients has yet to be ascertained.

The Central Role of the Macrophage

There are several lines of evidence suggesting that the cell type central in all responses to endotoxin, including the induction of tolerance to endotoxin, is the macrophage. The first and most obvious clue was that virtually all of the cytokine mediators now associated with LPS-induced toxicity were found to be macrophage products. However, a major key to the discovery of the true role of macrophages in endotoxicity was the spontaneous mutation of the C3H/HeJ mouse strain to a phenotype that was refractory to the administration of LPS (reviewed in Rosenstreich, 1985; Vogel, 1992). LPS-hyporesponsive mice were

found to have a single gene mutation on chromosome 4 (Watson *et al.*, 1978). Subsequently it was found that if lethally irradiated C3H/HeJ (Lps^d) or C3H/HeN (Lps^n) mice were transplanted with homologous or heterologous bone marrow cells, they survived to respond to LPS challenge in a manner consistent with the phenotype of the transplanted cells (Michalek *et al.*, 1980). In experiments with B-cell deficient, *xid* mice (Rosenstreich *et al.*, 1978) and with T-cell deficient athymic, *nu/nu* mice (Vogel *et al.*, 1979), it was shown that mice with either defect responded normally to LPS-induced toxicity only if they also had no mutation of the *Lps* gene, indicating that neither T nor B cells were critical in the determination of normal or deficient response to LPS. In 1981, Watanabe and Ohara showed that nuclei from splenocytes of non-responsive mice were perfectly capable of orchestrating a response to endotoxin when they were introduced into enucleated LPS-responsive cells by fusion (Watanabe and Ohara, 1981). Experimentation into the nature of the *Lps* gene defect also led to the discovery that C3H/HeJ mice failed to respond normally to LPS because they lacked a critical membrane component(s). Purified plasma membrane components from Lps^n cells, when fused with C3H/HeJ hyporesponsive B-cells, conferred normal LPS mitogenic responsiveness (Jacobovitz *et al.*, 1982). Most recently, Freudenberg *et al.* (1986) have determined in a galactosamine-sensitized mouse model that adoptive transfer of LPS-pretreated normal macrophages into C3H/HeJ mice caused them to behave as normally LPS-responsive mice, indicating that the defect in membrane components in the C3H/HeJ mouse strain is overcome by the addition of a normally responsive single cell type: the macrophage. It is the importance of the macrophage in the host response that makes study of this particular cell type *in vitro* an invaluable tool for the elucidation of the mechanisms of pleiotropic effects of LPS.

Historical Perspectives on the Induction of Endotoxin Tolerance

Before the turn of the century, William Coley, a surgeon from New York, observed that a patient, who had contracted a bacterial infection, responded not only with a high fever, but also with the regression of a solid tumor (Coley, 1893; reviewed in Beutler and Cerami, 1990). He reasoned that something about the invading bacterium had been the cause of both the fever and the tumor regression, and created an extract of killed *E. coli*, *S. marcescens*, and *S. pyogenes*, and gave it to cancer patients, hoping to help them reject their tumors. In 1936, Shear and Andervont analyzed Coley's extracts and isolated the active ingredient, which they called "bacterial polysaccharide" (Shear and Andervont, 1936). The practice of inducing "therapeutic febrile responses" for treatment of such diseases as typhoid led to the interesting observation that increasing amounts of the bacterial extracts were necessary on subsequent treatments to induce the same severity of fever as was achieved on first administration. Favorite and Morgan (1942; 1946) used an alcoholic extract of *Eberthella typhosa* (*Salmonella typhi*) to quantify the decrease in febrile response to a given dose of extract upon repeated administration. With the fever, they also noted severe leukopenia which was followed within several hours by leukocytosis accompanied by an increase in erythrocyte sedimentation rate. The increase in dosage necessary to induce a comparable febrile response subsequently was "non-immunologic," they surmised, since the production of circulating antibody was not required for the condition that they called "tolerance" to the toxic effects of the extract.

Exploration into the basis for the observed tolerance to the toxic bacterial extracts led Beeson (1946), after World War II, to confirm that humoral antibody response was not responsible for "pyrogenic tolerance," and that the phenomenon was also not specific to the O-antigen type of the bacteria, since

cross-tolerance to other serotypes of bacteria occurred. He found that the leukopenia noted by Favorite and Morgan occurred within the first hour of administration, and leukocytosis followed at between 4-8 hours (Beeson, 1947a). Tolerance was lost quickly, and normal responsiveness was restored by a rest of three weeks. Beeson found that antipyretics did not interfere with the induction of tolerance, and that it could not be transferred by passive administration of serum from a tolerant animal. He found that increasing an animal's core temperature mechanically did not induce tolerance. Beeson hypothesized that pyrogenic tolerance was mediated by the cells of the reticuloendothelial system (RES) by accelerated removal of the toxin from the circulation since "thorotrast," a thorium compound known to block RES activity, was able to cancel the effects of pyrogenic tolerance.

Morgan (1948) confirmed that pyrogenic tolerance was independent of specific circulating antibody, even when a purified phenolic extract of bacterial LPS, as it had come to be called, was administered to rabbits. The onset of tolerance was observed to occur within 24 hours, and responsiveness was at its lowest point on the third day after a single injection (Wharton and Creech, 1949). By the time the antibody response to a single injection was at its height at 21 days post-exposure, normal febrile responsiveness to another injection of LPS had returned.

The pathophysiologic effects of endotoxin were more recently discovered to be mediated by a series of proteinaceous, intercellular messenger molecules called cytokines, starting with the discovery of "endogenous pyrogen" (Atkins and Wood, 1955; reviewed in Atkins, 1960). A leap of understanding occurred when it was realized that the response to an injection of endotoxin was not immediate, but rather was delayed for a time (e.g., about 90 minutes for fever). The delay, Atkins and Wood thought, was due to production of an "endogenous

pyrogen" that was produced by the host's cells in response to injury by the toxin (Atkins and Wood, 1955; reviewed by Atkins, 1960). The "endogenous pyrogen," in turn, was responsible for the ensuing temperature dysregulation. In fact, they showed that an endogenous pyrogen in serum could be transferred to an individual rendered "tolerant," and it still retained the capacity to induce fever. They further hypothesized that tolerance must be due to an inability of tolerant individuals to produce endogenous pyrogen. Although no tolerance to the direct effects of endogenous pyrogen on the hypothalamus was found, tolerance due to decreased production of endogenous pyrogen was reversed by RES blockade (Beeson, 1947a). These findings implicated the cells of the immune system, and specifically cells of the monocytic lineage, in its induction.

Although tolerance during the first few days after exposure appeared to be due to the inability of the host to mobilize further production of endogenous pyrogens, Greisman and Woodward (1965) showed that there was a second, later stage of tolerance that was indeed anti-toxin antibody and O-antigen dependent, leading them to postulate two distinct immunologic mechanisms of resistance to the pyrogenicity of endotoxin. Endogenous pyrogen was subsequently found to be a product of mononuclear cells by Hahn *et al.* (1967). Kupffer cells in the liver were found to secrete an abundance of endogenous pyrogen when stimulated with endotoxin (Dinarello *et al.*, 1968). The secretion of endogenous pyrogen from Kupffer cells was inhibited in individuals made tolerant by previous exposure, even though mononuclear cells in the general circulation were not (i.e., they could still produce endogenous pyrogen). Therefore the liver was considered to be of premier importance in tolerance induction. The inability of Kupffer cells to secrete endogenous pyrogen during tolerance was indeed reversed by treatment of cells with thorotrast, and therefore, in 1970, Greisman and Woodward hypothesized that the early phase of tolerance was governed by

two factors. First, tolerance was due to an accelerated uptake of endotoxin by the Kupffer cells of the liver. Second, these cells were rendered refractory to further treatment with endotoxin and would not respond by secreting endogenous pyrogen, thus rendering the exposed individual tolerant.

As well as "endogenous pyrogen", which was later shown to be multiple cytokines induced by LPS (e.g., IL-1, TNF, IFN, IL-6; reviewed in Vogel and Hogan, 1990), endotoxin was found to induce the production of a number of other cell products. In 1971, Metcalf (Metcalf, 1971) found that bone marrow colony stimulating factors (CSF) were induced in response to endotoxin, and Quesenberry (1972) followed with the demonstration that the induction of tolerance resulted in a decrease in the amount of circulating CSF activity in the serum following LPS challenge. The induction of liver enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK) and tryptophan oxygenase, was also inhibited in animals rendered tolerant, and this inhibition was not due to a decrease in the activation state of the enzymes or due to their degradation upon tolerance induction (Rippe and Berry, 1972; 1973). Giron *et al.* (1973) showed that endotoxin also produces tolerance to the induction of interferons by LPS challenge, a phenomenon that had already been noted previously by Youngner and Stinebring (1965) and Ho (1967) in relation to enhancement of resistance to viral infections by LPS.

In summation of the knowledge to date concerning tolerance to endotoxin, Greisman and Hornick (1976) codified a list of characteristics of tolerance and differentiated between the "early" and "late" phases of endotoxin tolerance. Early phase tolerance developed within the first few hours after exposure to endotoxin, and was not specific for O-antigen type. The phenomenon was clearly not associated with the elaboration of anti-endotoxin antibodies, and was, in fact, inducible in hosts that were incapable of mounting an antibody response due to

splenectomy. Early phase tolerance, they found, was not transferable with plasma and could not be overcome by the transfusion of whole blood. It was not associated with general granulocytopenia and would develop even with detectable levels of circulating endotoxin still in the blood. After endotoxin clearance, unresponsiveness persisted, but was not due to the inability of cells to respond to preformed endogenous pyrogen. Although the early phase of tolerance resulted in an increased rate of clearance of endotoxin from the blood, the hyporesponsiveness was not due to an increase in the ability of the liver or plasma to inactivate LPS. Late phase tolerance, in contrast, required greater than 72 hours for initiation, and unlike the transient nature of early tolerance, persisted for weeks or months after a single exposure. This phase of tolerance was highly O-antigen specific and was directly correlated with the elaboration of anti-LPS antibodies. The late phase of tolerance was indeed transferable with serum (Greisman and Hornick, 1976).

The cellular mechanisms underlying early phase endotoxin tolerance remained elusive, however, and further research probed into the roles of the suspected contributory factors. Reitschel *et al.* (1980) and Dinarello and Bernheim (1981), demonstrated that macrophages cultured from animals rendered "tolerant" failed to secrete E series prostaglandins or IL-1, when restimulated with LPS i*n vitro*. Williams *et al.* (1983) described a parallel between the degree of depression of CSF production after a challenge dose of LPS *in vivo* and the duration of the hyporesponsive period due to a single LPS injection. Madonna and Vogel (1985) subsequently confirmed that tolerance was most profound three to four days after initial injection of LPS, and normal responsiveness returned in approximately a week. Madonna and Vogel also ascertained that endotoxin exposure brought about alterations in the bone marrow which resulted in an increase in the number and size of the available

macrophage precursor pool, and theorized that tolerance may, in part, be due to the inability of the immature cells to respond to LPS. Experiments with B-cell deficient, *xid* mice, T-cell deficient, *nu/nu* mice, as well as splenectomized mice, led Madonna and Vogel (1986) to the conclusion that neither B nor T cells were required for the development of tolerance. Interestingly, Madonna *et al.* (1986) also found that a dephosphorylated derivative of lipid A, monophosphoryl lipid A (MPL), could induce endotoxin tolerance, as shown by decrease in secreted CSF and IFN activities upon LPS challenge, and a significant increase the LD_{50} of subsequent doses of toxic LPS, although MPL was itself non-toxic (Ribi, 1984). The eventuality of a tolerance-inducing substance that was not toxic was something of a paradox, since LPS' toxic symptoms, as well as tolerance, were both felt to be cytokine-mediated.

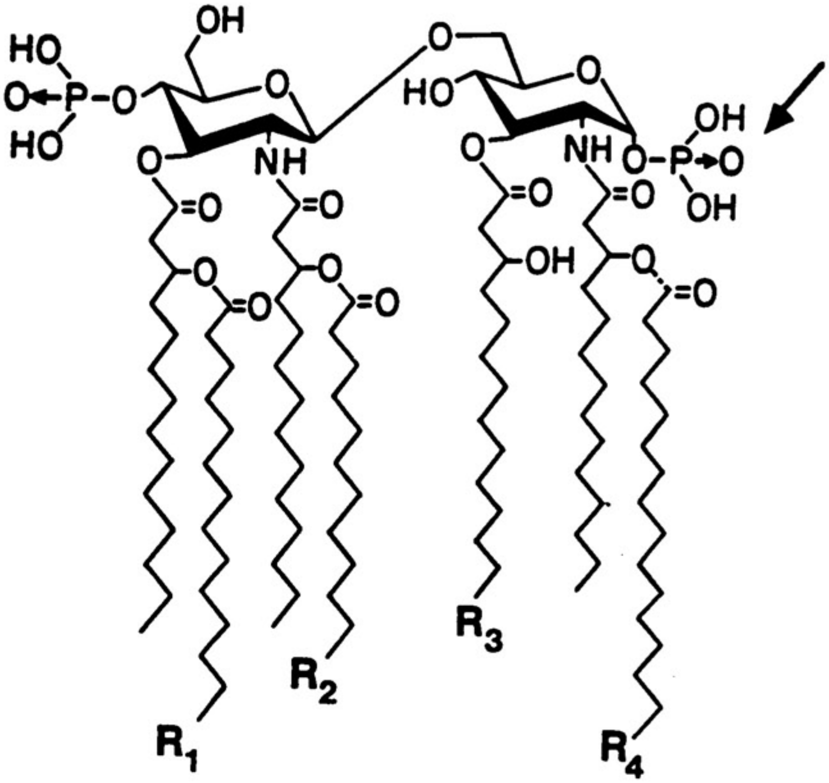
The importance of two cytokines, IL-1 and TNF in particular, in the induction of tolerance has been delineated by several means. In 1986, Gahring and Daynes compared the phenomenon of tolerance (which they called "desensitization") in UV-irradiated or LPS-stimulated macrophages. They showed that UV- and LPS-induced desensitization were dependent upon different mechanisms, but that desensitization in both cases was mediated, in part, by macrophage-derived IL-1. The involvement of TNF in tolerance was suggested originally by Ding and Nathan (1987), who showed that LPS-pretreatment precluded IFN-γ-activated macrophages from releasing TNF, and abrogated TNF's enhancement of respiratory burst activity stimulated by phorbol esters. Finally, Vogel *et al.* (1988) determined that a condition mimicking early endotoxin tolerance could be induced *in vivo* by co-injection of recombinant IL-1α and TNFα, but neither alone, and that combined action of the two produced the hematologic bone marrow changes previously associated with LPS-induced tolerance (Madonna and Vogel, 1985).

LPS Structure and Toxicity

Structurally, lipolysaccharide of the *Enterobacteriaceae* is an acylated, diphosphorylated, β-1,6-linked D-glucosamine disaccharide (referred to as lipid A) which is attached covalently to a core polysaccharide that consists of a series of unusual 7 (heptose) and 8 (2-keto-3-deoxy-D-mannooctulosonate) (KDO) carbon sugars (Westphal *et al.*, 1954) substituted with ethanolamine. The lipid A structure (Figure 1) is acylated at the 2 and 2' amino and 3 and 3' hydroxyl positions of the glucosamine rings with fatty acid groups of variable length. The number and length of the acyl groups is characteristic of a bacterial species (Luderitz *et al.*, 1971). The core polysaccharide, which is a relatively conserved structure within bacterial families, is linked enzymatically by the bacteria during LPS synthesis using a series of bacterial glucosyltransferase enzymes to repeating oligosaccharide side-chains known collectively as O-antigen (reviewed in Raetz, 1990). It is the O-antigen that confers antigenic and serological specificity on the bacterium (Luderitz *et al.*, 1982; 1987; reviewed in Brade *et al.*, 1988) and the sugar composition is, therefore, specific for a given "serotype." Lack of any of the required glucosyltransferases results in mutants with truncated LPS surface structures designated as bacterial chemotypes R_a through R_e (reviewed in Luderitz *et al.*, 1978). When interacting with the immune systems of higher animals, LPS acts as a T cell-independent B cell mitogen and macrophage activating factor (Morrison and Ulevitch, 1978). LPS is an amphipathic molecule, since the polysaccharide portion of the structure is highly hydrophilic and the lipid portion is extremely hydrophobic (Rietschel *et al.*, 1982). This duality of structure allows LPS to engage in both hydrophilic and hydrophobic

Figure 1. Chemical structure of diphosphoryl lipid A (DPLA). Lipid A is shown in a Fischer representation of the glucosamine disaccharide containing 2 and 2' N-linked β-hydroxy myristic fatty acid groups at R2 and R4 and 3 and 3' O-linked C14 myristate groups at R1 and R3 in the acylation pattern common to *E. coli* and *Salmonella* species. Appended in acyloxyacyl linkage from R1 and R2 are myristate (C14) and laurate (C12) groups, respectively. Attached at R4 by a dotted line is a C16 palmitate residue that is present in approximately 20% of *Salmonella minnesota* lipid A and absent in *E. coli* lipid A, as well as in 80% of *S. minnesota* lipid A. Phosphoryl groups are positioned at C1 of the reducing terminal glucosamine and C4' of the non-reducing terminal glucosamine. KDO residues and core polysaccharides in natural LPS are attached at C6 of the non-reducing terminus. The arrow indicates the position of the phosphoryl group that is removed in the monophosphoryl lipid A (MPL) used in these studies.

Adapted from Takada, H., and Kotani, 1989.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interactions with host cell components. In solution, LPS has been shown to form micelles and bilayered laminar structures with fatty acids aligned toward the hydrophobic interior (Shands, 1971; Bara *et al.*, 1973; Esser *et al.*, 1979). The amphipathic nature of LPS makes its interaction with the cellular components of the host immune system highly varied.

In order to dissect the relationship of structure to function, many structurally distinct varieties of LPS were isolated and compared for activity in an extensive panel of biologic effects such as complement activation, mitogenicity, pyrogenicity, and lethality (Luderitz *et al.*, 1978). Luderitz and others concluded that a complete lipid A structure was necessary for demonstration of the full range of LPS' toxic effects. In addition to lipid A, lipid A substructures were also isolated and purified (Rick and Osborn 1977; Rick *et al.*, 1977; Nishijima and Raetz, 1979; Takayama *et al.*, 1983; Qureshi *et al.*, 1985; Raetz *et al.*, 1985). In 1981, it was demonstrated that lipid A could be biochemically synthesized (Inage *et al.*, 1981; Galanos *et al.*, 1984) and that the synthetic material was as active as purified, natural lipid A (Kotani *et al.*, 1985; Takada and Kotani, 1989; Luderitz *et al.*, 1989; Takayama *et al.*, 1990). In addition, certain synthetic substructures have proven relatively non-toxic and these are being scrutinized for potential use as adjuvants, immunostimulants, or antagonists to LPS-induced toxicity (Ribi, 1984; Proctor *et al.*, 1986; Kovach *et al.*, 1990; Lam *et al.*, 1991; Stutz *et al.*, 1991). For example, Ribi found that mild acid hydrolysis of LPS from *Salmonella* resulted in a monophosphoryl compound (MPL) that was 100 - 10,000-fold less toxic than native LPS, but that retained many of the beneficial attributes of lipid A, such as its ability to serve as an adjuvant, and to stimulate tumoricidal activity against solid tumors (Ribi, 1984; Ribi *et al.*, 1986).

On the basis of pyrogenicity, local Schwartzman reaction (Ishikawa, *et al.*, 1991), B-cell mitogenicity (Takayama *et al*, 1990), induction of interferon and tumor necrosis factor (Keiner *et al.* ,1988), activation of macrophages to release prostaglandins (Luderitz *et al.*, 1989), lethal toxicity *in vivo,* as well as *Limulus* amebocyte lysate assay *in vitro,* Rietschel *et al.* (1987), confirmed the structural portions of lipid A required for maximal toxicity by using synthetic analogues of lipid A. The glucosamine disaccharide lipid A structure containing the 2 and 2'-N-linked and 3 and 3'-O-linked fatty acids plus the presence of acyloxyacyl groups appended from the 2' and 3'-linkedfatty acids (a total of 6 fatty acid groups), was required for maximal bioactivity. Greater than or fewer than 6 fatty acid groups, resulted in lowered ability to induce toxic symptoms, as was noted by direct comparison of the toxicity of synthetic *E. coli* lipid A (compound 506), a hexaacyl structure containing 2' and 3'-acyloxyacyl groups, and synthetic *S. minnesota* lipid A (compound 516), a heptaacyl structure containing three acyloxyacyl groups (Rietschel *et al.*, 1987). Structural requirements for B-cell mitogenesis and positive *Limulus* amoebocyte lysate assay were less strict, since acylation patterns made little difference in these tests. The phosphate groups at the 1 and 4' positions of the lipid A also were found to be important in toxicity, as removal of one or both large hydrophilic moieties resulted in decreased toxicity as well as a stepwise decrease in solubility after the removal of each group.

Rietschel *et al.* (1987) also correlated the removal of the phosphoryl groups from the lipid A structure (that resulted in fewer toxic characteristics) with a change in the three-dimensional conformation of the appended fatty acid groups, a notion that was reinforced by the finding that the phase transition temperature from solid to liquid of the monophosphoryl compounds was higher than that of the corresponding diphosphoryl derivative. A similar inverse relationship was found between the phase transition temperature and the ability

of LPS partial structures to activate the release of arachadonic acid intermediates (Luderitz *et al.*, 1989). Using a multitude of bioactivity measurements, such as lethal toxicity in chick embryos and in galactosamine pre-treated mice, IFN-inducing activity, and stimulation of macrophage IL-1 production, Galanos *et al.* (1984) and Takada and Kotani (1989) determined that the toxicity of synthetic lipid A derivatives (Kotani *et al.*, 1985; Galanos *et al.*, 1986) could be represented in a relative hierarchy of toxicity as follows: diphosphoryl lipid A (LAPP) > 1 monophosphoryl lipid A (LAHP) > 4' monophosphoryl lipid A (LAPH) > dephosphorylated lipid A (LAHH), and that the most toxic was the *E. coli*-like hexaacyl lipid A with similar length 12 carbon fatty acids. Loppnow *et al.* (1989) also showed that IL-1 was induced in a hierarchy by LPS partial structures, where smooth *Salmonella* LPS induced IL-1 more efficiently than synthetic hexaacyl or heptaacyl lipid A which, in turn, induced more IL-1 than lipid A partial structures. Core oligosaccharides and oligoacyl lipid A followed with even lower ability to induce IL-1. In another series of studies correlating the biologic activity of lipid A with its structure, Baker *et al.* (1992) showed that the disaccharide backbone of lipid A with at least five acyl groups was absolutely required for the abolition of T suppressor cell activity. The 4' position phosphoryl group was found important in stimulation of polyclonal B cell activation, which is a hallmark of *in vitro* LPS effects.

Therapeutic possibilities for Endotoxic Shock

Rapid increase in knowledge of the toxic effects of LPS at both the cellular and molecular levels has led to a recent proliferation in the number of possible avenues for therapy of septic shock. The new therapeutic possibilities can be divided into five groups, according to the underlying rationale of each. Many studies are currently underway to examine the potential of LPS-binding proteins,

LPS antagonists, antagonists of LPS-induced toxic factors, antibodies directed against LPS or toxic factors induced by LPS, and immunomodulatory agents in the treatment and prevention of septic shock.

Among the binding proteins that are currently being considered for therapeutic use are endotoxin neutralizing protein (ENP) (Wainwright, 1991) and a cationic protein called CAP 37 (Larrick *et al.*, 1991). ENP is isolated from the hemolymph of *Limulus polyphemus* (horseshoe crabs), and neutralizes endotoxin by binding LPS very tightly. Although probably not of much use for injection into humans (because of the prompt antibody response it would evoke), the protein may be bound to fibrous sheets in a filtration system to be used for extracorporeal removal of endotoxin from the circulation in a manner similar to kidney dialysis (Wainright, 1991). CAP 37 is a PMN-derived protein that is a reactive nitrogen inhibitory peptide. It is an enzymatically-inactive protein that resembles several well known serine proteases such as elastase and cathepsin G in structure. This protein inhibits LPS-induced nitrogen radical production and TNF release by macrophages, and may be effective for the amelioration or prevention of LPS-induced tissue injury (Larrick *et al.*, 1991).

Several LPS-derivatives and novel varieties of LPS that are devoid of bioactivity in human cells are currently being tested for their therapeutic value as LPS antagonists. Lipid IV_A (Rick *et al.*, 1977; Golenbock *et al.*, 1991) and *Rhodopseudomonas (Rhodobacter) sphaeroides* lipid A (Strittmatter *et al.*, 1983) are among those in the category of LPS-antagonists that have been shown to antagonize LPS-induced effects by blocking the binding of toxic LPS to target cells (Takayama *et al.*, 1989; Kirkland *et al.*, 1991; Qureshi *et al.*, 1991). These compounds have been shown to block LPS-induced IL-1, TNF, IL-6, and PGE_2 secretion from human macrophages *in vitro* and fail to induce these toxic

cytokine intermediates either *in vivo* or *in vitro* (Golenbock *et al.*, 1991; Kovach *et al.*, 1990; Qureshi *et al.*, 1991).

Substances that can act as LPS-induced toxic factor antagonists are attractive as potential therapeutic agents since they may be used even if infection is already established. For example, recombinant IL-1-receptor antagonist (rIL-1ra), is a human macrophage-derived protein. In baboons, it shortened the time of hypotension and blocked the autocrine and paracrine effects of IL-1, as well as reduced mortality from endotoxic shock (Ohlsson *et al.*, 1990). The rIL-1ra has also been effective in Phase I and Phase II clinical trials as an anti-inflammatory agent for bowel, joint, pulmonary, and systemic inflammation, though it has no known agonist activity itself (Thompson *et al.*, 1991). It is now undergoing Phase III clinical trials (Donnelly, personal communication). Other factors, such as antagonists of arachadonic acid metabolites (Smith *et al.*, 1988) and platelet activating factor (PAF) (Braquet *et al.*, 1989), may be useful in reducing thrombocytopenia, and leukotriene- and thromboxane-mediated hemodynamic changes associated with LPS-induced damage, as well as capillary damage due to the synergistic interactions of TNF and PAF.

Monoclonal anti-LPS and anti-cytokine antibodies are currently being tested in Phase II clinical trials to determine their efficacy in reducing mortality from endotoxic shock. Three prototype antibodies are under close scrutiny: (i) those that are of murine origin, (ii) those that are of human origin, (iii) and those that are chimeric murine/human constructs. The anti-LPS antibody, E5, is a monoclonal IgM anti-LPS antibody of murine origin, that has been found to bind lipid A, ReLPS, and MPL. Phase I clinical trials of this antibody showed that its half-life was 18 - 19 hours in the circulation. Twenty-four hours after a 2 mg/kg dose, the blood level was > 5 μg/ml. In Phase II clinical trials, of 468 patients with Gram negative septicemia to receive the drug, 47% developed anti-E5

antibodies, which is a major drawback to this treatment approach. E5 was especially effective in increasing the resolution of organ failure in patients without shock, increasing the survival of this group to 74% (Gorelick *et al.* 1991). If patients were in shock, however, E5 treatment was of no survival benefit.

Another anti-LPS antibody under testing is HA-1A, a monoclonal human IgM anti-lipid A antibody (Ziegler *et al.*, 1991). Phase I clinical trials showed that this antibody was safe at doses up to 300 ng, and in Phase II clinical trials, 543 patients with symptoms of sepsis at 24 medical centers around the U.S.A., Canada, and in Europe, received 100 ng HA-1A each, with no adjustment for weight. Of those, 200 patients subsequently had positive blood cultures for Gram negative sepsis. Elaboration of anti-HA-1A antibodies by patients was not a major problem due to its human origin. Treatment with HA-1A (105 of 200 patients) showed a reduction in mortality of patients with septicemia (39%). The reduction in mortality was greater in patients in shock (42%), and greatest for patients in both shock and organ failure (51%) (Ziegler *et al.*, 1991). The significance of this finding is highly controversial.

Chimeric human/murine anti-TNF antibodies also show potential in the treatment of septic shock. One such antibody, designated CA2, is a chimeric monoclonal IgG antibody and has a murine hypervariable region with human constant regions. It has been shown to block IL-6 secretion from fibroblasts and to decrease adherence of neutrophils to vascular endothelial cells *in vitro* (Smith *et al.*, 1991). Another anti-TNF antibody, called CBP571, has been shown to decrease circulating levels of IL-8, neutrophil elastase, bilirubin, lactate (which indicates an ameliorating effect on metabolic acidosis), and to bring about an increase in the mean arterial pressure in baboons infused with LPS (Bodmer *et al.*, 1991).

The last category of therapeutic agents for septic shock are those where the substance administered is immunomodulatory of the entire response to LPS. The non-toxic LPS derivative MPL is under examination for use as an immune stimulant (Beatty *et al.*, 1991). MPL is relatively non-toxic, and if given in advance, has been shown to reduce LPS-induced toxic effects and induce endotoxin tolerance (Madonna *et al*, 1986; Chase *et al.*, 1986). It has been shown to be an effective adjuvant in increasing antibody response to other antigens (Ribi *et al.*, 1986; Hiernaux *et al.* 1989). In mice, pretreatment with MPL decreased toxic response to subsequent infection and increased survival of mice from intraperitoneal bacterial challenge with $1 - 2 \times 10^8$ *E. coli* (Chase *et al.*, 1986). In Phase I and II clinical testing, MPL was used as prophylaxis against wound sepsis in head and neck cancer patients who required additional complex surgical resection and/or flap reconstructions (Beatty *et al.*, 1991). Fifteen patients were treated with IV infusion of 100 $\mu g/m^2$ of MPL 48 hours prior to surgery. Results showed that MPL was safe for use as a prophylactic immune stimulant, since only four patients developed post-operative infections, but that there may be problems with wound healing, as four other patients developed flap inviability. Other clinical trials using MPL are also underway (Ribi ImmunoChem, Inc., 1991).

Purpose of the Study

The purpose of this research was to use the different bioactivities of LPS, MPL, and its derivatives, as tools in the murine model of endotoxic shock and endotoxin tolerance to study the cellular and molecular mechanisms which underlie endotoxin sensitivity. The research was divided into three sections, selected to incorporate observations from different vantage points. At the

organismal level, studies *in vivo* were utilized to quantify responses to LPS as they were affected by structural variation of the bioactive LPS-derivatives and the presence of response modifying substances. At the cellular level, tissue culture methods were used to correlate macrophage responses *in vitro* to those observed in the organism *in vivo*. Finally, a study of gene transcription in macrophages stimulated by LPS or its derivatives was carried out to compare the relative abilities of LPS and its partial-structures to induce gene expression.

MATERIALS AND METHODS

Mice. Five to six week old female mice were used for all experiments reported herein. C57BL/6J, C3H/OuJ and C3H/HeJ mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and female C3H/HeN mice were obtained from NCI, NIH (Frederick, MD). Mice were used within one week of receipt. Mice were housed in a laminar flow hood, were maintained under 12 hour light and dark cycles until use, and were fed autoclaved standard lab chow and acid water *ad libitum*.

Reagents.

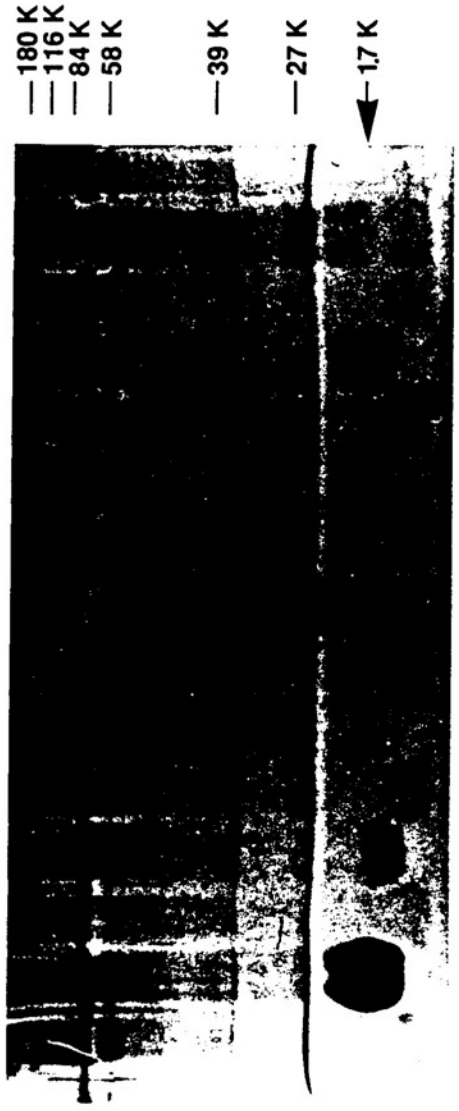
LPS and related reagents. Protein-free (<0.008%), phenol/water-extracted *Escherichia coli* K235 lipopolysaccharide (LPS) was prepared by the method of McIntire *et al.* (1967). *Salmonella minnesota* LPS R595 (ReLPS), *S. minnesota* diphosphoryl lipid A (DPLA), and *S. minnesota* monophosphoryl lipid A (MPL), and, *Salmonella typhimurium* MPL were purchased from Ribi ImmunoChem Research, Inc. (Hamilton, MT) (Ribi, 1984). Synthetic *E. coli* MPL (Compound 504) was purchased from ICN Corp. (Cleveland, OH). *E.coli* D31M4 ReLPS was the generous gift of Nilofer Qureshi (University of Wisconsin at Madison, WI). Stock solutions of LPS, ReLPS, DPLA, and MPL were prepared at 1 mg/ml in normal saline containing 0.2% triethylamine and alternately were sonicated vigorously and heated to 65° C in a water bath to increase solubility.

To confirm the expected molecular weights of the LPS preparations, polyacrylamide gel electrophoresis was carried out according to the method of Maniatis *et al.* (1983). For comparative size determinations of *E.coli* K235 LPS and *S. minnesota* R595 derivatives, 50 µl of each material at 1 mg/ml was suspended

in 50 µl of Laemmli loading buffer, pH 6.8, plus 2 µl 0.1% bromophenol blue, and mixed thoroughly. Samples were boiled for 10 minutes prior to loading. Twenty µl of each sample compound (10 µg) was loaded into the lanes of a 6% "stacking gel" which was overlayed onto a 25% SDS-PAGE "running gel," and samples were electrophoresed at 8V/cm in a 10% SDS-Tris-glycine continuous buffer system until the dye front reached the running gel. Current was then increased to 15 V/cm for separation in the running gel. SDS gels were stained with Rapid-Ag-Stain (ICN Corp., Cleveland, OH) silver stain and were maintained in 2% glycerol for photography (Figure 2).

The pentaacylated form of *R. sphaeroides* DPLA (RsDPLA), and its MPL derivative (RsMPLA) were generously isolated and purified for this study by Nilofer Qureshi (Middleton Memorial Veteran's Hospital, Madison, WI). RsDPLA and RsMPLA were isolated and purified as follows: LPS was first extracted from *R. sphaeroides* ATCC 17023 cells by the phenol/chloroform/petroleum ether method (Galanos *et al.*, 1969). RsDPLA was prepared by suspending crude LPS at 3 mg/ml in 0.02 M sodium acetate, adjusting the pH to 2.5 with acetic acid, and incubating for 70 min at 100° C and recovered as described by Qureshi *et al.* (1983). Crude RsDPLA was purified by passage through a DEAE-cellulose acetate column using a continuous gradient of 0.03 - 0.08 M ammonium acetate in chloroform/methanol/water (2:3:1, v/v/v), and fractions were analyzed via silica gel thin layer chromatography as described elsewhere (Qureshi *et al.*, 1991). *R. sphaeroides* monophosphoryl lipid A (RsMPLA) was prepared from the LPS as previously described (Qureshi *et al.*, 1983). Like the MPL from *S. minnesota* and *S. typhimurium*, this species also lacks the reducing terminal phosphoryl group. The RsMPLA pentaacyl fraction was purified by thin layer chromatography on silica gel H in a solvent system of

Figure 2. SDS-PAGE electrophoresis for comparative size determinations of *E.coli* K235 LPS and *S. minnesota* R595 LPS derivatives. Ten μg of each LPS derivative was electrophoresed on 25% SDS-PAGE and stained with silver stain (Rapid-AG-Stain, ICN Radiochemicals, Irvine, CA) as follows: **lane 1**. *S. minnnesota* ReLPS; **lane 2**. Synthetic *E. coli* MPL (LA-15-PH); **lane 3.** 3' deacylated *S. minnesota* MPL; **lane 4**. *E. coli* K235 LPS; **lane 5**. *S. minnesota* MPL; **lane 6**. *S. minnesota* DPLA; **lane 7.** Low Molecular Weight Standards (BioRad, Richmond, CA). **Lane 4** shows an LPS "ladder" due to variable length oligosaccharide O-antigen of smooth *E. coli* LPS. Lanes 1-3 and 5,6 show near molar equivalence at approximately 1.7 - 1.8 kDa size, since material in the wells migrated equivalently for structures not containing O-antigen side chains.



1 - *S. minnesota* ReLPS
2 - LA-15-PH Synthetic *E. coli* MPL
3 - 3' Deacylated *S. minnesota* MPL
4 - *E. coli* K235 LPS
5 - *S. minnesota* MPL
6 - *S. minnesota* DPL
7 - Molecular Weight Standards

chloroform/methanol/water/ammonia (50:25:4:2, vol:vol) (Qureshi *et al.*, 1988). For experimental use, *Rhodopseudomonas sphaeroides* derivatives were reconstituted from the lyophilized state with pyrogen-free saline and sonicated to insure solubilization prior to use. Stock solutions were maintained at -20° C until they were used.

Specimen preparation for electron microscopy. The physical state of LPS and MPL in solution and the ability of both to interact with biological membranes was assessed under physiologic conditions by electron microscopy of SRBC stroma that had been treated with LPS and MPL. Samples for embedding were prepared according to the method of Shands (1971). Two milliliters of washed sheep red blood cells (SRBC) were incubated with either buffer only or 1 mg/ml LPS or MPL for 1 hour in 0.05 M Tris buffer pH 7.5 plus 2% BSA. Cells were sedimented by centrifugation and then were lysed in 0.01 M Tris hypotonic lysis buffer at pH 6.8. Free hemoglobin was removed by repeated washing of the SRBC stroma with 0.2 M Tris buffer at pH 7.5. Membranes were then resuspended in 500 µl of 2% OsO_4 in 0.1 M Tris buffer at pH 7.5 for 15 minutes to fix the stroma. The stroma were removed from osmium tetroxide solution by washing with fresh buffer, then were resuspended in 2% BSA in 0.5 M Tris at pH 7.5. Sample material was then allowed to gel in the tube by adding 1 drop of 25% glutaraldehyde. Tubes were sliced with a razor and fragments of the coagulum were teased apart. Coagulum samples were progressively dehydrated by passage through solutions of increasing ethyl alcohol concentration (50% to 75% to 95%) for 10 min each. Samples were subsequently treated with three changes of absolute ethanol for 10 min, cleared in 2 changes of propylene oxide for 10 min each, and imbedded in Epon 812. The resin was cured at 60° C for one week

prior to sectioning by USUHS professional services. Electron microscopy was performed on a JEOL 100 Cxr electron microscope.

Cytokine antagonists. Recombinant IL-1 receptor antagonist (rIL-1ra) (Batch # 8908) was generously supplied by Robert Thompson of Synergen, Inc. (Boulder, CO). The recombinant protein was diluted in pyrogen-free saline (Abbott Laboratories, North Chicago, IL) for injection. Stock solutions of rIL-1ra at 44.5 mg/ml were aliquoted to prevent repeated freezing and thawing and rIL-1ra was frozen at -70° C prior to use.

Rabbit anti-TNFα and control IgG antibody preparations were provided by Edward Havell of Trudeau Institute (Saranac Lake, NY) (North and Havell, 1988) and were reconsitituted from lyophilized form in pyrogen-free saline prior to use. Neutralizing activity of this reagent was determined in a standard TNF cytotoxicity assay (Vogel and Hogan, 1991) and activity is expressed as neutralizing units per milliliter. Control rabbit IgG antibody dosage was adjusted to reflect an equivalent dose by weight.

Protocols for *In Vivo* Studies.

Initial Injection Protocol. Mice were injected intraperitoneally with 0.25 ml pyrogen-free saline as a control, or the indicated concentrations of LPS or MPL in 0.25 ml saline at time zero. Individual groups of mice were bled from the retroorbital plexus at the indicated times post-injection for the establishment of the initial time course. Thereafter, mice were typically bled at one peak time that was most appropriate for the particular cytokines being tested. Pooled blood samples were allowed to clot for 30 minutes at room temperature, and were centrifuged at maximum speed in a tabletop microfuge (Beckman, Irvine, CA). The separated serum samples were frozen at - 20° C until testing was performed.

In all cases, glucose testing was performed on serum samples immediately after thawing, to preclude deterioration of serum glucose levels. In some cases, results for C57BL/6J and C3H/HeN mice have been pooled because they were indistinguishable from each other. Results for *S. minnesota* and *S. typhimurium* MPL were also pooled, since the two varieties of MPL tested were indistinguishable for all parameters tested.

In vivo Tolerance Induction Protocol. Previous studies have shown that administration of a sublethal dose of LPS results in a markedly decreased capacity to respond to a challenge injection of LPS 3 to 4 days later by production of CSF (Madonna *et al.*, 1985; Williams *et al.*, 1983). Induction of early endotoxin tolerance was carried out by dividing mice in groups of three to eight mice per treatment group per experiment. Mice were injected intraperitoneally with 0.25 ml of pyrogen-free saline, or the indicated concentrations of LPS, ReLPS, DPLA, or MPL in 0.25 ml of saline on Day 0. The concentrations of LPS and MPL used were established previously (Madonna *et al.*, 1986) and were confirmed for the multiple lots of MPL used for this study. A 200 μg dose of MPL consistently led to tolerance equivalent to that induced by 25 μg of LPS, as assessed by inhibition of LPS-induced CSF (Williams *et al.*, 1983). Three days later (Day 3), one group of saline-inoculated mice was reinoculated with saline, and the other groups were "challenged" with 25 μg of LPS in 0.25 ml (approximately 0.1 LD_{50}). At the indicated times after challenge injection, all mice were bled from the retroorbital plexus. Pooled blood samples were allowed to clot for 30 minutes at room temperature, and were centrifuged at maximum speed in a tabletop microfuge (Beckman, Irvine, CA). The separated serum samples were collected and frozen at - 20° C until testing was performed.

Recombinant IL-1 receptor antagonist and anti-TNFα antibody protocols. To test the efficacy of rIL-1ra and anti-TNFα antibody in blocking LPS-induced effects *in vivo*, groups of 4 to 5 mice (per treatment per experiment) were injected intraperitoneally with the indicated combinations of pyrogen-free saline (Abbott Labs, North Chicago, IL), rIL-1ra, anti-TNFα or control rabbit IgG, and/or *E. coli* K235 LPS (25 μg/mouse) or *S. minnesota* MPL (200 μg/mouse). Anti-TNFα or control antibody, diluted in saline, were given five hours prior to LPS in all experiments. Unless otherwise indicated, rIL-1ra was given simultaneously with LPS. Dilutions of rIL-1ra and LPS were also prepared in pyrogen-free saline. Six hours after injection, mice were bled and the sera pooled and assayed for CSF activity and serum glucose levels, as described below. To assess the efficacy of the rIL-1ra and/or anti-TNFα antibody to block LPS-induced early endotoxin tolerance, mice were injected on Day 0 with saline, LPS, MPL and/or the rIL-1ra, or anti TNFα antibody, and the mice were challenged three days later (Day 3) with saline or 25 μg LPS. Again, the ability to respond to LPS to produce CSF was used as an indicator of LPS responsiveness.

Assays for Cytokine Activity.

Measurement of CSF activity in serum. Serum was tested for CSF activity in a bone marrow colony assay in semisolid agar exactly as described previously (Madonna and Vogel, 1985,1986; Madonna *et al.*, 1986; Vogel *et al.*, 1987). Serum samples were tested for CSF activity as follows: Bone marrow cells obtained from tibias and femurs of C3H/HeJ (Lps^d) mice were pooled and processed by gradient density centrifugation in Lymphocyte Separation Medium (LSM; Litton Bionetics, Kensington, MD). Cells from the gradient interface were collected and diluted to 1×10^5 cells per ml in a mixture of tissue culture medium containing 15% fetal calf serum and molten agar. One milliliter of the cell suspension was

added to each well of a six-well tissue culture plate (Costar, Cambridge, MA.) which contained 0.2 ml serial dilutions of test serum per well. Each sample was assayed in duplicate. The contents of the wells were mixed by swirling and were allowed to solidify. Cultures were incubated at 37° C and 6% CO_2 for 6 to 7 days. Colonies containing >25 cells were enumerated with a dissecting microscope. CSF activity, expressed as units per milliliter, was calculated by multiplying the number of colonies per well by the reciprocal of the final dilution of the sample. Only colony numbers on the linear portion of the dilution curve were used to calculate CSF concentration. Serum samples were assayed in duplicate at each dilution tested.

IFN assay. IFN activity was determined for pooled serum samples by a modification (Vogel *et al.*, 1991) of the method of Rubinstein *et al.* (1981). Serial two-fold dilutions of serum samples were prepared in 50 µl volumes in flat-bottomed 96-well culture dishes (Falcon Plastics, Oxnard, CA). To each well was added 50 µl of L929 fibroblasts (1×10^5 cells per well). After a 24 hour incubation at 37° C and 6% CO_2, the supernatants were aspirated, and the cells were infected with 100 µl of vesicular stomatitis virus (Indiana strain) per well in medium containing 10% fetal calf serum, at a multiplicity of infection of 0.1. At 18-24 hours post-infection, the cells were washed three times with cold Earle's balanced salt solution and fixed for 10 minutes with 5% formalin solution. The cells were stained for 5 minutes with 0.05% crystal violet in 20% ethanol, and the plates were subsequently washed with tap water and allowed to air dry. Before the plates were read, stain was eluted with 100 µl of methanol per well and the absorbance at 595 nm was read in a BioTek EL 308 enzyme-linked immuno-sorbent assay (ELISA) reader (BioTek Instruments, Inc., Winooski, VT). Three controls were included in each assay: (i) uninfected, medium-treated L929 cells

as a cell control; (ii) virus-infected, medium-treated L929 cells as a virus control; and (iii) a titration of 100 U of National Institutes of Health (NIH) mouse fibroblast IFN reference standard (G-022-904-511; Research Resourses Branch, National Institute of Allergy and Infectious Diseases) per milliliter. The first well in the dilution series of the sample which exhibited an optical density equal to that of the virus control wells (i.e., no IFN protection) was defined as the endpoint. Reciprocal titers, expressed as units per milliliter, were based on the titration of the NIH standard.

TNF assay. TNF activity in serum samples was determined by a standard cytotoxicity assay with actinomycin-D-treated fibroblasts (Hogan and Vogel, 1991). For TNF assay, 100 µl of 4×10^5 L929 cells per ml was plated in each well of 96-well microtiter plates (Falcon Plastics, Oxnard, CA) and incubated overnight to form monolayers. Following this, the medium was aspirated and 50 µl of fresh medium was added to each well. A test sample (50 µl) was then added to the first well, and 10 two-fold serial dilutions were made in the remaining wells. Finally, 50 µl of actinomycin D (8 µg/ml; Sigma Chemical Co., St. Louis, MO) was added to each well. Plates were incubated for 18 hours at 37° C and 6% CO_2. At the end of the assay period, plates were washed with normal saline, stained with 0.05% crystal violet in 20% ethanol for 10 minutes, rinsed in tap water, and air dried. Stain was eluted with 100 µl absolute methanol and the absorbance of each well was read at 595 nm in a Biotek EL 380 microtiter plate reader (Biotek Instruments, Inc., Winooski, VT). Units of TNF are presented as the reciprocal of the highest supernatant dilution which resulted in 50% lysis of a well multiplied by the dilution factor (i.e., 20) to give a measure of TNF activity in units per milliliter. The lower and upper limits of sensitivity of the assay are

80 U/ml (i.e., 4 U/50 µl) and 40,960 U/ml (i.e., 2048 U/50 µl), respectively (Vogel and Hogan, 1991).

IL-6 assay. IL-6 activity was measured in a hybridoma cell proliferation assay described in detail elsewhere (Aarden *et al.*, 1987; VanSnick *et al.*, 1986) at the laboratory of William Benjamin of Hoffmann-LaRoche, Inc. (Nutley, NJ) For the IL-6 assay, B-9 hybridoma cells were treated with serial 10-fold dilutions of test sera, and proliferation was allowed to occur for 72 hours. Proliferation was measured by incorporation of tritiated thymidine for the last 18 hours of culture. IL-6 activity is also expressed as units per milliliter. An IL-6 unit is defined as the reciprocal of the final dilution of sample which induces half-maximal proliferation of the hybridoma cells.

IL-1 assay. IL-1α protein levels were quantified by means of a commercially available ELISA assay which utilizes a specific monoclonal anti-murine IL-1α antibody to detect the presence of this cytokine (Genzyme Immunologicals, Cambridge, MA). All reagents were provided with the assay kit. A 96 well plate coated with anti-IL-1α antibody was first blocked with a protein-based buffer, and serum samples were diluted 1:4 in the wells with serum diluent. A standard curve was prepared by including dilutions of a mouse recombinant IL-1α standard. Standards and samples were incubated in the wells for 40 min at 37° C. The wells were subsequently washed and treated with biotinylated anti-mouse IL-1α antibody. Bound IL-1α was detected using an avidin-horseradish peroxidase detection system. Absorbance of the test wells was read at 450 nm. IL-1 α protein levels are represented in pg/ml and were calculated by regression analysis of the standard curve. Individual serum samples were assayed in duplicate.

Measurement of blood glucose levels. Blood glucose levels were determined for serum samples with a glucose oxidase reagent kit (Sigma Chemical Co., St. Louis, MO) in a modified spectrophotometric microassay (Henricson *et al.*, 1990). A standard curve was prepared each time the assay was performed by diluting glucose standard solution (Sigma Chemical Co., St. Louis, MO). Standards ranged from 20 to 100 mg/dl and were treated identically to the test samples. Twenty-five microliters of test samples and standard solutions were diluted 1:5 with 100 µl of deionized water; then, 20 µl of each diluted sample was placed into each of six replicate wells of a 96-well plate (Falcon Plastics, Oxnard, CA). To each well, 200 µl of glucose oxidase enzyme-color reagent was added. A 20 µl amount of deionized water in each of six wells served as a blank. Plates were incubated for 30 minutes at 37° C before the absorbance at 450 nm was read. The mean absorbance of the six wells for each sample was used to calculate the glucose concentration of the sample using linear regression analysis of the standard curve.

Protocols for *In Vitro* Studies.

Macrophage isolation and culture. Five to six week old LPS-responsive (*Lps*^{n}) C57BL/6J or C3H/OuJ mice (Jackson Laboratory, Bar Harbor, ME) were used as the source of macrophages for all *in vitro* studies. Thiglycollate elicited peritoneal exudate macrophages were isolated by peritoneal lavage with ice cold sterile physiologic saline 4 days after intraperitoneal injection of 3 ml of sterile 3% thioglycollate broth (BBL, Becton-Dickinson, Cockeysville, MD). Cells were washed and resuspended in RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM Hepes, 0.3% sodium

bicarbonate, and 2% heat inactivated fetal calf serum and cultured at 2 x 10^5 cells per well in 96 well tissue culture plates (Costar, Cambridge, MA) at 37° C and 6% CO_2. For all RNA studies, 5 ml of 2 x 10^6 cells/ml of resuspended cells were plated in 66 mm culture dishes (Costar, Cambridge, MA) and were allowed to adhere overnight. Non-adherent cells were removed by washing twice with fresh medium 20 - 24 hours after plating and the remaining adherent cells were cultured as indicated. Cells were treated with medium or the indicated concentrations of LPS or LPS-derivatives at 37° C in 6% CO_2. At the indicated times, culture supernatants were harvested and then were stored at -20° C until assayed for TNF activity or supernatants were aspirated and the cells subjected to RNA isolation as described below. For the experiments in which *Rhodopseudomonas sphaeroides* derivatives were used as antagonists, macrophages were pretreated with *R. sphaeroides* derivatives for two hours prior to the addition of medium only or the indicated LPS derivatives without washing out the RsDPLA or RsMPLA. Cultures were incubated for an additional 20 hours at 37° C in 6% CO_2. Culture supernatants were then harvested for TNF bioassay.

In vitro tolerance assay. The assay for induction of *in vitro* tolerance is essentially identical to that originally described by Virca *et al.* (1989). To assess the induction of hyporesponsiveness to LPS (e.g., *in vitro* tolerance), macrophages were first cultured identically as for the production of TNF. Following removal of supernatants from cultures after 20 hours of incubation, the cells were washed twice with fresh media and re-cultured for one hour prior to "challenge" with either medium only or 10 ng/ml *E. coli* K235 LPS. Cells were reincubated for 20 additional hours, at which time supernatant media was again harvested for assay of TNF activity. Samples for TNF assay were frozen at -20° C prior to determination of TNF activity. For RNA studies, TNF was determined in

culture supernatants at 4 hours of "challenge" and the cells were lysed in 4 M guanidine isothiocyanate (Fluka Chemika-BioChemika, Ronkonkoma, NY) for preparation of total cellular RNA (See below).

Isolation of total cellular RNA. Macrophages were lysed with 4 M guanidine isothiocyanate and total cellular RNA was isolated by the cesium chloride gradient centrifugation method of Chirgwin *et al.* (1979). Northern blot analysis was carried out by the method of Maniatis *et al.* (1983) as follows: For separation of RNA species, 5 μg of total RNA from each experimental condition was subjected to 1% formaldehyde-agarose gel electrophoresis, and total RNA was transferred to Nytran filters (Schleicher & Schuell, Inc., Keene, NH) by means of capillary action in 10 X SSC. Filters were rinsed briefly to remove extraneous salt and were dried for 15 minutes prior to UV crosslinking for 2 minutes. Filters were hybridized overnight with random-primed ^{32}P-labeled cDNA probes after a 4 hour prehybridization at 42° C. Probes utilized for these experiments were as follows: a 1100 bp segment of the cDNA of TNFα (Pennica *et al.*,1985), kindly provided by Dr. Bruce Beutler (Howard Hughes Med. Inst., Dallas, TX); a cDNA probe specific for murine β–actin (Tokunaga *et al.*,1986), provided by Dr. Michael Prystowsky (University of Pennsylvania, Philadelphia, PA); a cDNA encoding bases 275 - 1329 of IL-1 β (Ohmori *et al.*,1990); a 450 base cDNA segment of murine IP-10 (Ohmori and Hamilton, 1990); and specific cDNA probes for four other LPS-inducible genes (i.e., D2, D3, D7 and D8) first described by Tannenbaum *et al.* (1988) as strongly induced by LPS within 4 hours. Filters were hybridized 15 - 18 hours at 42° C in sealed mylar bags. After hybridization, filters were washed twice at room temperature for 20 minutes in 2X SSC containing 0.1% sodium dodecyl sulfate and once at 65° C for 15 minutes in 0.1X SSC containing 0.1% sodium dodecyl sulfate. Northern blot filters were

exposed to Kodak XAR-5 film with intensifier screens for 4 - 24 hrs at -70° C and subsequently were used to expose blank PhosphorImager screens. Exposed PhosphorImager screens were analyzed using PhosphorImager software in a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Exposed X-ray film was analyzed using a scanning densitometer (Xerox, Corp., Rochester, NY) and NIH-Image software kindly provided by Dr. Wayne Rasband, NIH, Bethesda, MD. Between successive hybridizations, Northern blots were stripped of radioactive probes by boiling in DEPC-treated distilled water for 5 minutes. Between hybridizations blots were stored at -20° C to preclude RNA decomposition. Gene expression on all Northern blots was normalized to the expression of β-actin, by dividing the PhosphorImager volume measurement of each gene by the volume measurement for β-actin in the same lane (gene/β-actin ratio). A measurement comparable to the PhosphorImager volume was created for scanned blots by multiplying the computer measured area of the expressed band by the measured band's intensity. The relative expression for each gene was then internally normalized to the expression of that same gene stimulated by 10 ng/ml LPS for four hours (by dividing the gene/β-actin ratio for each gene by gene/β-actin ratio of the 10 ng/ml LPS challenge), for the dose response and *in vitro* tolerance experiments, or to the maximal expression of 1000 ng/ml LPS within the first 12 hours for the time course experiments (100%).

Acyloxyacyl hydrolase assay. Acyloxyacyl hydrolase (AOAH) enzyme activity (Hall and Munford, 1983; Munford and Hall, 1985) in macrophage cytosolic extracts was determined as follows. LPS responder (Lps^n) peritoneal exudate cells from either C3H/OuJ or C57BL/6J mice were cultured at 2×10^5 cells per well in 96-well microtiter plates for measuring AOAH enzyme activity in lysates

of stimulated cells (Falcon Plastics, Oxnard, CA) and incubated at 37° C and 6% CO_2 overnight. After twenty hours incubation, non-adherent cells were removed by washing twice with fresh medium. Cells were then incubated with medium only or stimulated with the indicated concentrations of *E. coli* K235 LPS or *S. minnesota* MPL and incubation was resumed for twenty hours, at which time cell supernatants were aspirated and after rinsing with PBS, cells in each well were lysed in 100 µl PBS plus 0.1% Triton-X-100 (Sigma Chemical Co., St. Louis, MO), containing 2mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co., St. Louis, MO). Plates were placed on a mechanical shaker for 10 minutes to aid lysis and then the plates were centrifuged at 2000 rpm for 10 minutes to sediment cell debris. Aliquots of cytoplasmic supernatant extracts from identical treatment wells were pooled and frozen at -20° C for AOAH assay (Hall and Munford, 1983; Munford and Hall, 1985; 1986). AOAH enzyme assays were kindly performed by David Ginkel, in the laboratory of Robert S. Munford (University of Texas Health Science Center, Dallas, TX).

To measure deacylation of LPS by AOAH in intact cells, rather than in cell lysates (as described above), peritoneal exudate macrophages were cultured at 1.5×10^6 cells per well in 6 well plates (Falcon Plastics, Oxnard, CA) for 20-24 hours as indicated above. Non-adherent cells were removed by washing twice with fresh medium. Cells were treated with medium only or 100 ng/ml *E. coli* K235 LPS for 20 hours. After incubation, cells were washed twice with fresh medium and were restimulated with either medium alone or 10 ng/ml *E. coli* K235 LPS. After an additional 20 hours incubation, cells were again washed twice with fresh medium and were pulsed with 10 ng/ml LPS ^{3}H-LPS (Specific activity = 4×10^6 dpm/µg). Tritiated *S. typhimurium* PR 122 (galE nag-) LPS (containing ^{3}H only in the fatty acid portion of the LPS structure) was the generous gift of Robert S. Munford (University of Texas Health Science Center,

Dallas, TX). At the end of a six hour incubation with LPS, plates were washed and LPS was replaced by fresh medium and the incubation was continued. At 10 hours after addition of the ^{3}H-LPS, supernatants were aspirated and cells were lysed in 0.05% Triton-X-100 (Sigma Chemical Co., St. Louis, MO) at 4° C. Lysates were frozen at -70° C prior to extraction of the free fatty acids. Aliquots of 250 µl per sample were adjusted to 25 mM NaCl and 200 µg of purified bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO) per sample was added to bind all remaining non-deacylated LPS. Absolute alcohol was added to a final concentration of 70% (vol:vol) in a volume of 1 ml to precipitate LPS-BSA complexes. After thorough mixing, samples were frozen at -70° C for 1 hour and then were centrifuged at 10,000 x g for 10 minutes to sediment the precipitate. Supernatants were counted in a Beckman scintillation counter (Irvine, CA) with standardized quench corrections. Raw dpm/sample measurements were corrected by subtracting counts extracted from wells containing no cells that had been treated identically to treatment groups. Rate of deacylation was calculated in pg/ml/hour from the specific activity of the ^{3}H-LPS and the corrected dpm/ml for each sample.

ELISA protocol for competitive inhibition of rabbit anti-5D3 (anti-80 kDa LPS-receptor protein) antibody binding. Peritoneal exudate macrophages were plated at 2 x 10^{5} cells per well in a 96 well plate and were allowed to adhere overnight in suplemented RPMI 1640 plus 2% fetal calf serum at 37° C in 6% CO_2. Prior to assay, cells were washed twice with fresh medium. LPS and MPL in 10% Earle's balanced salt solution (EBSS) plus 10% fetal calf serum and 0.02% sodium azide were added to triplicate rows for each concentration tested. Cultures were incubated with LPS and MPL for 1 hour at 37° C in 6% CO_2. Affinity-purified rabbit anti-5D3 antibody (Rα5D3) was provided by David

Morrison (University of Kansas Medical Center). At the end of 1 hour, Rα5D3 (25 μg/ml) (Chen *et al.*, 1990; 1992), control rabbit IgG (Cappell, West Chester, PA) (25 μg/ml), or medium only were added to one row each of the triplicate rows of LPS- and MPL-treated macrophages, and equilibration was allowed to occur for 45 min at 4° C. Then, wells were washed three times with cold EBSS plus 1% fetal calf serum, and 100 μl of a horseradish peroxidase-conjugated goat-anti-rabbit IgG (BioRad, Richmond, CA) secondary antibody (1: 2,000 dilution) in EBSS plus 10% fetal calf serum was added to each well. Plates were reincubated for 45 min at room temperature, washed 5 times with fresh EBSS plus 1% fetal calf serum prior to detection of antibody binding via addition of the substrate and hydrogen peroxide (Falk *et al.*, 1988). Absorbance was read at 490 nm. Binding of secondary antibody alone was used as an assay control, and specific binding of the Rα5D3 was corrected for each LPS and MPL concentration by subtraction of the binding of the irrelevant antibody. No difference was found between the competition performed on cells treated with iodoacetic acid or sodium azide (data not shown).

RESULTS

INDUCTION OF EQUIVALENT EARLY ENDOTOXIN TOLERANCE BY LPS AND MPL *IN VIVO*

Monophosphoryl lipid A (MPL), a derivative of lipid A (Figure 2), is dephosphorylated at position one of the reducing-terminal glucosamine moiety (Rietschel *et al.*, 1987), and has been shown to be significantly less toxic than either lipid A or intact LPS (Raetz *et al.*, 1983; Ribi, 1984; Rietschel *et al.*, 1987). Even though modified by the removal of the phosphoryl group from the reducing terminal glucosamine residue, MPL retains the ability to induce several of the beneficial effects of LPS administration. MPL can act as an excellent adjuvant (Ribi *et al.*, 1986; Baker *et al.*, 1992) and not only stimulates polyclonal B-cell activation, but abolishes antigen-specific T suppressor cell activity (Hiernaux *et al.*, 1989). MPL improves survival from lethal irradiation (Madonna, personal communication) and has been shown to stimulate monokine production *in vivo* (Ribi *et al.*, 1986; Madonna *et al.*, 1986a; Keiner *et al.*, 1988) and to facilitate tumor regression *in vivo* (Ribi, 1984).

The term "endotoxin tolerance" was first used to describe a period following administration of LPS during which animals failed to respond to subsequent injections of LPS (Favorite and Morgan, 1942). "Early" endotoxin tolerance is observed after a single sublethal injection of LPS, and peaks three to four days after initial injection (Williams *et al.*, 1983). Animals gradually regain normal responsiveness approximately eight days after the initial LPS injection. LPS is cleared from the serum during the period of hyporesponsiveness, and

tolerance cannot be transferred to fully responsive animals via anti-LPS antibodies in the serum of LPS tolerant animals (Beeson, 1946, 1947a, 1947b; Wharton and Creech, 1949).

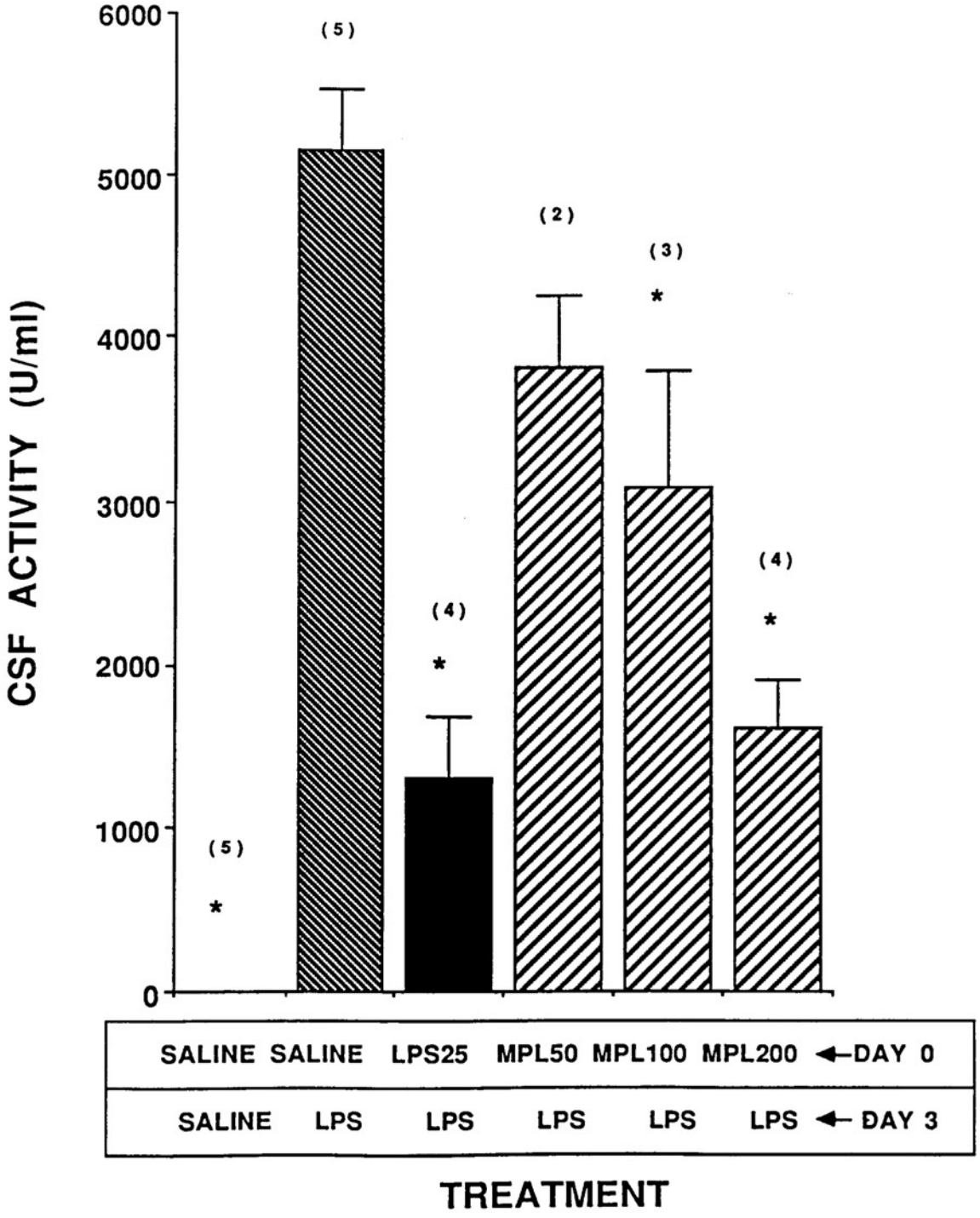
Macrophages derived from tolerant animals were found to produce reduced levels of endogenous pyrogen (Atkins and Wood, 1955; Dinarello *et al.*, 1986) and prostaglandins of the E series (Rietschel *et al.*, 1980; Dinarello and Bernheim, 1981; Dinarello and Wolff, 1978; Knudsen *et al.*, 1986) when stimulated with LPS *in vitro*. Not only is early endotoxin tolerance inducible with serologically unrelated types of LPS, but also with the non-toxic lipid A derivative MPL, although significantly more MPL than LPS is required to elicit the same degree of tolerance (Madonna *et al.*, 1986). Treatment of mice with a combined regimen of recombinant Interleukin 1 (rIL-1α) and recombinant Tumor Necrosis Factor (rTNFα), results in a state of hyporesponsiveness which mimicked "early endotoxin tolerance," as evidenced by decreased production of Colony Stimulating Factor (CSF) activity in response to a challenge injection of LPS three days after the administration of cytokines (Vogel *et al.*, 1988).

Since both LPS and MPL have been shown to induce early endotoxin tolerance, as measured by an equivalent reduction in CSF production or an increase in LD_{50} following LPS challenge (Madonna *et al.*, 1986), there appeared to be a paradox: If both toxicity and tolerance were cytokine-mediated, and both MPL and LPS induced early endotoxin tolerance, then why is MPL less toxic? In these experiments, equivalent tolerance-inducing doses of LPS and MPL were established and the serum levels of cytokines induced by these doses of LPS *versus* MPL were compared. Doses of LPS and MPL which resulted in equivalent tolerance were chosen to compare LPS- and MPL-induced effects.

The cytokine panel tested was expanded to include CSF, TNF, IFN, IL-6, and IL-1α, in addition to LPS-induced hypoglycemia.

Induction of Tolerance by LPS *versus* MPL. Previously, it was shown that MPL could induce early endotoxin tolerance in outbred mice, although a significantly higher dose was required to achieve the same degree of tolerance when compared with intact LPS (Madonna *et al.*, 1986). For this study, we utilized inbred mice to minimize genetic variation in LPS responsiveness. Inbred mice were treated on Day 0 (D0) with saline, 25 μg LPS, or 50 μg, 100 μg, or 200 μg MPL. On Day 3 (D3) one group of mice from the saline pretreated group was challenged with saline and the rest were challenged with 25 μg LPS, and six hours after the challenge injection serum samples were collected for CSF determination. The six hour optimal time point and 25 μg concentration of LPS challenge were based upon previous studies using Swiss (ICR) mice (Madonna and Vogel, 1985; 1986; Madonna *et al.*, 1986), and both parameters were confirmed in preliminary studies in inbred mice. Figure 3 shows the results of a series of experiments in which LPS and MPL were compared for their ability to induce early endotoxin tolerance in inbred mice, as assessed by production of CSF after LPS challenge. Using either C57BL/6J or C3H/HeN mice, a significantly higher dose of MPL was required to induce the same degree of tolerance as observed when 25 μg *E. coli* LPS was administered as the tolerance-inducing agent. Twenty-five μg of LPS administered on Day 0 reduced the level of CSF produced in response to a challenge injection of 25 μg LPS on Day 3 to 26% of the saline-pretreated control group. Two hundred μg of MPL was found to reduce CSF upon LPS challenge equivalently, to 31% of control levels. Doses of 50 and 100 μg MPL induced only partial tolerance, as evidenced by suboptimal

Figure 3. Tolerance induction by LPS *versus* MPL as assessed by CSF production. Mice (3-8 per treatment group per experiment) were injected on Day 0 (D0) with Saline (SAL), LPS (25 μg), or MPL (50 μg, 100 μg, or 200 μg). On Day 3 (D3), one group of saline pretreated mice was reinjected with saline and the others were "challenged" with LPS (25 μg). Six hours after the D3 injection, mice were bled and sera were assayed for CSF activity (U/ml) as described in the Materials and Methods. Treatments are indicated on the X-axis. Results represent the arithmetic means +/- the standard error of the mean of (n) separate experiments, where (n) is the number of experiments for the indicated treatment. Asterisks indicate treatment groups that are significantly different from the Day 0 SAL/Day 3 LPS treatment group by unpaired Student's *t* test ($p < 0.05$). To evaluate statistical significance by a more sensitive method, the data for Figure 3 were subjected to analysis by the method of Least Squares Means comparison, by Dr. Lawrence Douglas, University of Maryland statistician. This method creates weighted means for treatments of unequal (n) in an incomplete block design of experiments, to overcome the relative advantage or disadvantage of a smaller or larger (n) for any treatment. When the data were treated in this manner, $p= 0.0158$ for 50 μg MPL, $p= 0.0014$ for 100 μg MPL, and $p= 0.0001$ for 200 μg MPL pretreatment. In each case, p value measured by ANOVA method was lower than that measured by Student's *t* test, alleviating the concern that statistical significance might be claimed in error by use of the unpaired *t* test. Although relatively insensitive, the Student's t test was retained as the statistical measure of significance in all subsequent assays.

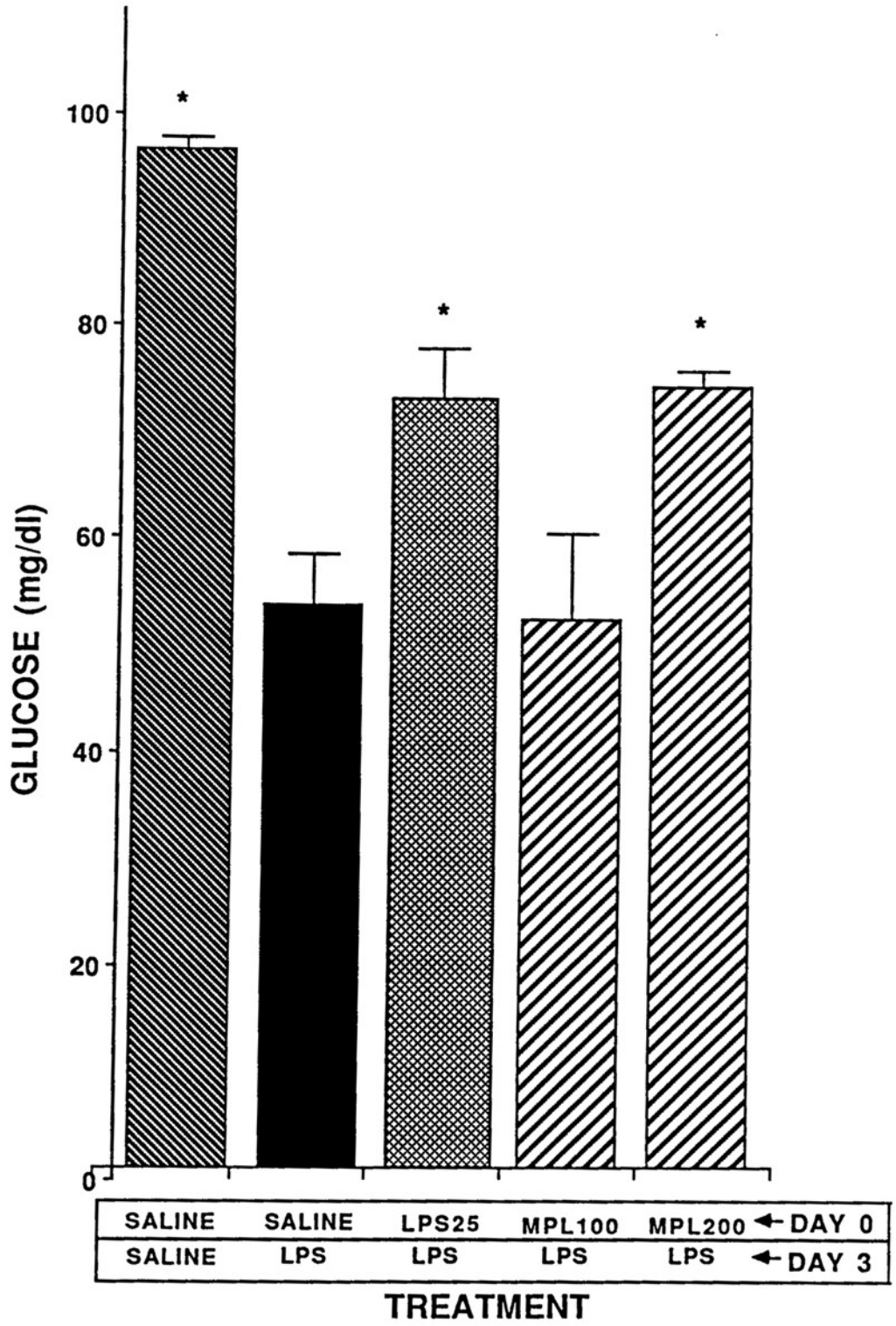

6000
5000
4000
3000
2000
1000
0
CSF ACTIVITY (U/ml)
(5)
(4)
(2)
(3)
(4)
(5)
*
SALINE SALINE LPS25 MPL50 MPL100 MPL200 ←DAY 0
SALINE LPS LPS LPS LPS LPS ← DAY 3
TREATMENT

reduction of CSF activity. Tolerance induction data for LPS and MPL in C3H/HeN and C57BL/6J mice were not statistically different, nor where results using MPL derived from *S. typhimurium or S. minnesota* (data not shown). Thus, using the induction of CSF as an indicator of LPS responsiveness, we confirmed the previous findings of Madonna *et al.* (1986) that significantly more MPL was required (200 μg) to induce the same level of tolerance as induced by 25 μg LPS.

Another measure of LPS-induced toxicity is the induction of hypoglycemia (Berry, 1977). The capacity of LPS *versus* MPL to mitigate LPS-induced hypoglycemia upon challenge was also compared (Figure 4). Challenge with LPS on Day 3 induced severe hypoglycemia in saline pre-injected controls, reducing the serum glucose by 45% six hours after injection. In mice pre-treated with either 25 μg LPS or 200 μg MPL, the decrease in serum glucose in response to LPS challenge on Day 3 was only 24 % when experimental values were compared ($p \leq 0.05$). As was observed for CSF (Figure 3), subtolerizing doses of MPL (100 μg) were found not to protect mice from the LPS challenge-induced hypoglycemia (Figure 4). For all subsequent experiments, the response of animals made tolerant with 200 μg MPL or 25 μg LPS were compared at the indicated times after challenge injection with 25 μg LPS.

LPS-induced IFN, TNF, and IL-6 activities were also compared in mice pre-treated on Day 0 with LPS *versus* MPL. Serum IFN levels were measured at two time points, 2 hours (optimal) and 6 hours (declining) based upon previous time course studies with outbred and inbred mice (reviewed in Vogel, 1992). In these studies, IFN levels in inbred mice were found to be consistent with results in outbred mice in that they were higher in saline-pre-treated controls at 2 hours following LPS challenge than at 6 hours. Although both LPS and MPL induced tolerance to a subsequent LPS challenge, MPL was not as effective as LPS in reducing the amount of post-challenge IFN antiviral activity detected.

Figure 4. Tolerance induction by LPS and MPL as assessed by serum glucose levels. Mice (3-8 per treatment group per experiment) were injected with Saline (SAL), LPS (25 μg), or MPL (100 μg or 200 μg) on Day 0 (D0). On Day 3 (D3), one group of saline-pretreated mice was reinjected with saline, and the others were "challenged" with LPS (25 μg). Six hours after the D3 injection, mice were bled and sera were assayed for serum glucose content (mg/dl), as described in the Materials and Methods. Treatments are indicated on the X-axis. Results represent the arithmetic means +/- the standard error of the mean of 3 - 5 separate experiments. Asterisks indicate treatment groups that are significantly different from the Day 0 SAL/Day 3 LPS treatment group by unpaired Student's *t* test ($p < 0.05$).


GLUCOSE (mg/dl)
100
80
60
40
20
0
*
*
*
SALINE
SALINE
LPS25
MPL100
MPL200
DAY 0
SALINE
LPS
LPS
LPS
LPS
DAY 3
TREATMENT

IFN levels were significantly higher in MPL-pretreated mice than those made tolerant by LPS at 2 hours, but not at 6 hours post-challenge (Figure 5). This finding is consistent with the lesser efficacy of MPL as compared with LPS as a tolerogen for LPS-induced IFN in outbred animals (Madonna *et al.*, 1986).

When tolerance induction by LPS and MPL was compared with respect to post-challenge levels of circulating TNF, LPS and MPL were also found to induce tolerance with respect to TNF production (Figure 6). TNF production peaked one hour post-challenge injection, and in both the LPS- and MPL-tolerized groups, production of TNF was reduced significantly below the levels produced in the group pre-treated with saline. This reduction in TNF activity in the serum of tolerant animals was even more striking at two hours following LPS challenge and, unlike the tolerance associated with IFN production, both LPS (25 μg) and MPL (200 μg) were comparably tolerogenic. In two separate experiments, IL-6 levels were also compared in LPS- and MPL-tolerized mice following challenge injection. Both LPS and MPL inhibited production of IL-6 induced by LPS challenge equivalently (Table I).

When a commercially prepared ELISA specific for murine IL-1α became available within the last 6 months, IL-1α levels were also compared in serum of LPS- *versus* MPL- pretreated mice. Until this assay became available, bioactivity of IL-1 in serum could not be measured, due to the presence of non-specific inhibitors (Oppenheim, 1986; Oppenheim *et al.*, 1991). Also, it should be noted that unlike the bioassay for IL-1 activity, this ELISA employs a monoclonal antibody specific for IL-1α . Thus, this assay detects the presence of IL-1α only, and not IL-1β (see Materials and Methods). As was seen for CSF, IFN, TNF, and hypoglycemia, pretreatment of mice with 200 μg MPL reduced circulating levels of IL-1α induced by LPS "challenge" to approximately the same level as pretreatment with 25 μg LPS, reflected as a reduction of 24.5% and 21.2% of

Figure 5. Tolerance induction by LPS and MPL as assessed by production of IFN activity. Mice (3-8 per treatment group per experiment) were injected with saline, LPS (25 μg) or MPL (200 μg) on Day 0 (D0). On Day 3 (D3), one group of saline pretreated mice was reinjected with saline, and the others were "challenged" with LPS (25 μg). At two and six hours after the D3 injection, mice were bled and sera were assayed for IFN activity (U/ml) as described in the Materials and Methods. The results represent the geometric mean and the error bars are the 95% confidence limits for 5-7 separate experiments per treatment.

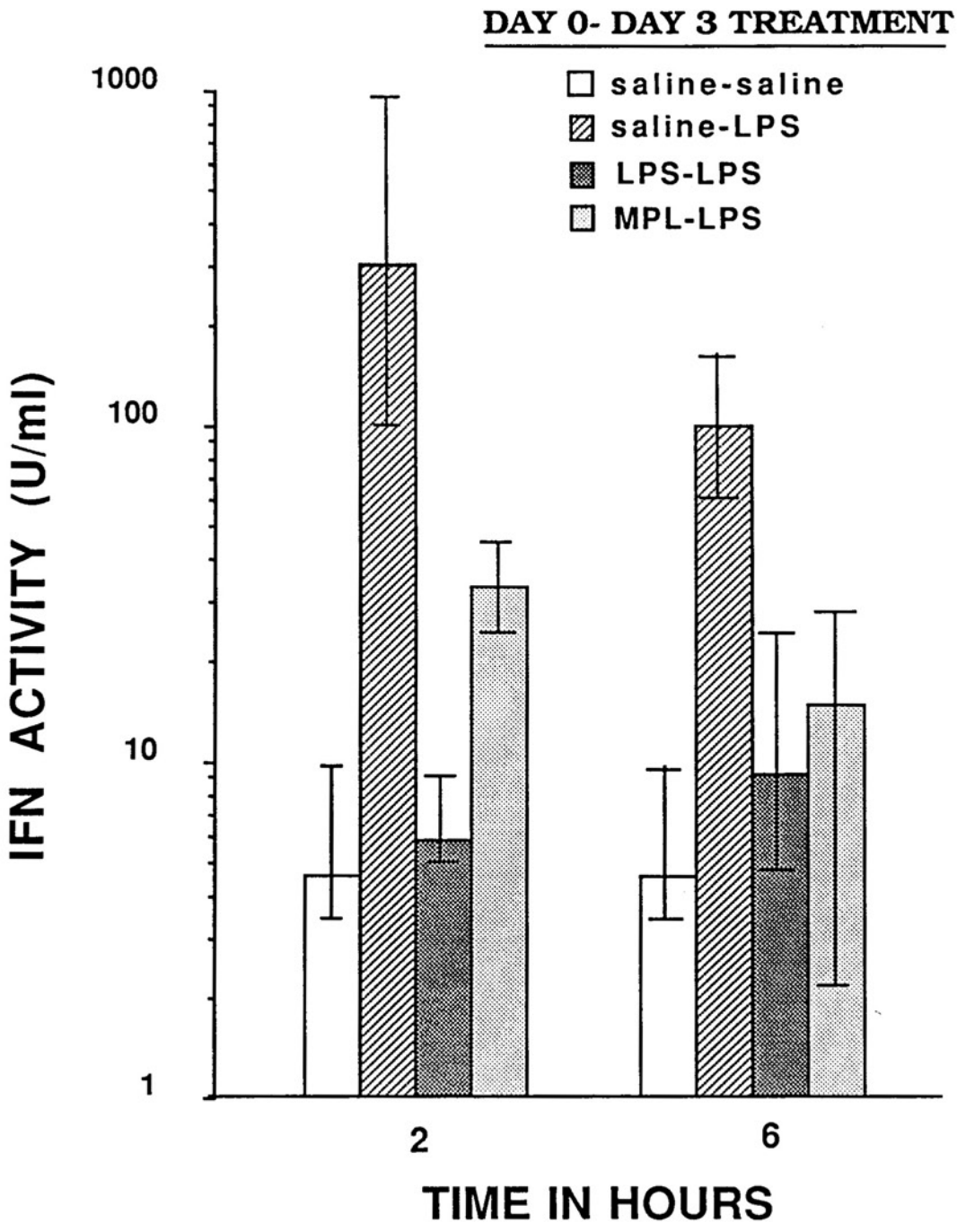

DAY 0- DAY 3 TREATMENT
saline-saline
saline-LPS
LPS-LPS
MPL-LPS
1000
100
10
1
IFN ACTIVITY (U/ml)
2
6
TIME IN HOURS

Figure 6. Tolerance induction by LPS and MPL as assessed by production of TNF activity. Mice (3-8 per treatment group per experiment) were injected on Day 0 (D0) with saline, LPS (25 μg), or MPL (200 μg). On Day 3 (D3), one group of saline pretreated mice was reinjected with saline and the others were "challenged" with LPS (25 μg). At one and two hours after the D3 injection, mice were bled, and TNF activity (U/ml) was assayed as described in the Materials and Methods. The results represent the geometric mean and the error bars are the 95% confidence limits for 6-10 separate experiments.

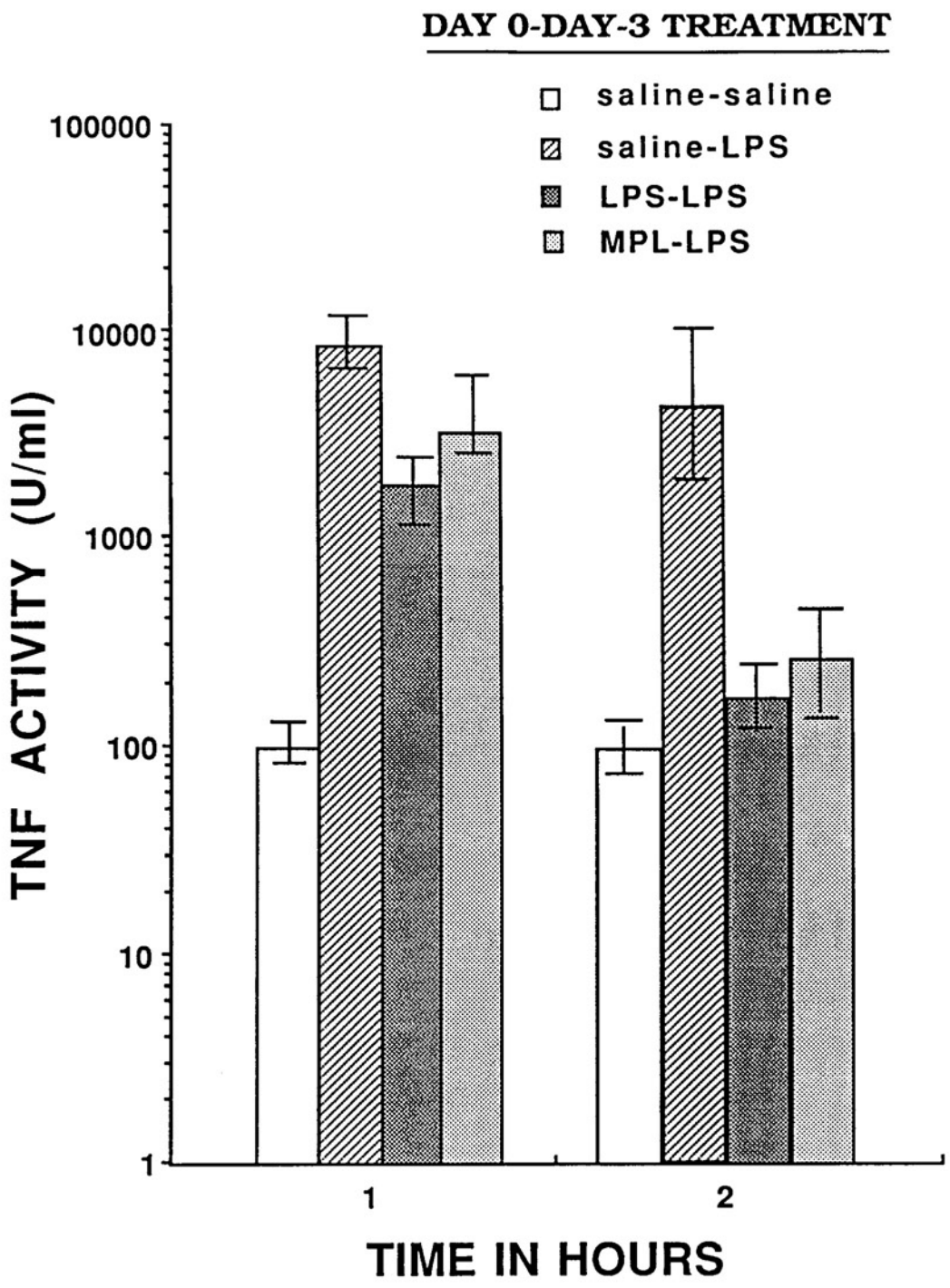

DAY 0-DAY-3 TREATMENT
saline-saline
saline-LPS
LPS-LPS
MPL-LPS
100000
10000
1000
100
10
1
TNF ACTIVITY (U/ml)
1
2
TIME IN HOURS

Table I.

Induction of Tolerance byLPS *versus* MPL as Assessed by Production of IL-6 Activity

Treatment[a] D0 - D3	IL-6 Activity (U/ml)	
	Experiment 1	Experiment 2
Saline - Saline	< 100	< 100
Saline - LPS	25,600	19,000
LPS - LPS	600	1,200
MPL-LPS	600	1,200

[a]Mice (4 per group) were injected on Day 0 (D0) with LPS (25 μg) or MPL (200 μg). Four hours after challenge injection, serum was collected and assayed for IL-6 activity, as described in the Materials and Methods. The results were derived from two separate experiments.

control IL-1α for LPS and MPL, respectively (Figure 7).

Doses of LPS and MPL given to mice were measured in micrograms per mouse. Since the molar ratio of smooth LPS is not equivalent to that of MPL, due to the lack of O-antigen oligosaccharides, core sugars, KDO, and the phosphoryl group in MPL, the observed differential dosage for MPL *versus* LPS to induce tolerance was examined. As seen in Figure 2, polyacrylamide gel electrophoresis confirmed the presence of a "ladder" in our smooth LPS preparation, indicating that a substantial proportion of our LPS contained varying lengths of O-antigen. Since only a small fraction of the LPS structure by weight is actually lipid A, the disparity between the tolerance-inducing activity of LPS *versus* MPL might conceivably be much larger than 8-fold. Therefore, to compare MPL to derivative structures that were more similar to MPL in molar ratio, but still contained complete lipid A, ReLPS (e.g., KDO_2-lipid A) and DPLA (Figure 1) were also used in order to compare doses necessary to induce an equivalent degree of tolerance to 200 μg MPL and 25 μg LPS. When *S. minnesota* ReLPS and DPLA were used to induce tolerance, 25 μg of each induced a comparable depression of CSF activity to pretreatment with 25 μg of LPS or 200 μg of *S. minnesota* MPL (Figure 8).

To address the possibility that preparations of natural MPL were of uncertain purity and of heterogeneous acylation, a homogeneously pure, synthetic preparation of hexaacyl *E.coli* MPL (Compound 504) (see Materials and Methods) was purchased to compare with chemically isolated *S. minnesota* MPL for tolerance-inducing ability. The synthetic MPL was found to induce a degree of tolerance (with respect to CSF induction) that was indistinguishable from that induced by the natural preparations of *S. minnesota* MPL used thus far. The CSF induced upon challenge of saline-pretreated mice with 25 μg of LPS was significantly greater than CSF induced in animals pretreated with 25 μg LPS,

Figure 7. Tolerance induction by LPS and MPL as assessed by production of IL-1α. Mice (3 per treatment group per experiment) were injected on Day 0 (D0) with Saline (SAL), LPS (25 μg), or MPL (200 μg). On Day 3 (D3), one group of saline pretreated mice was reinjected with saline and the others were "challenged" with LPS (25 μg). Four hours after the D3 injection, mice were bled and sera were assayed for IL-1α (pg/ml) as described in the Materials and Methods. Results represent the arithmetic mean +/- the standard error of the mean of duplicate determinations from 2 separate experiments.

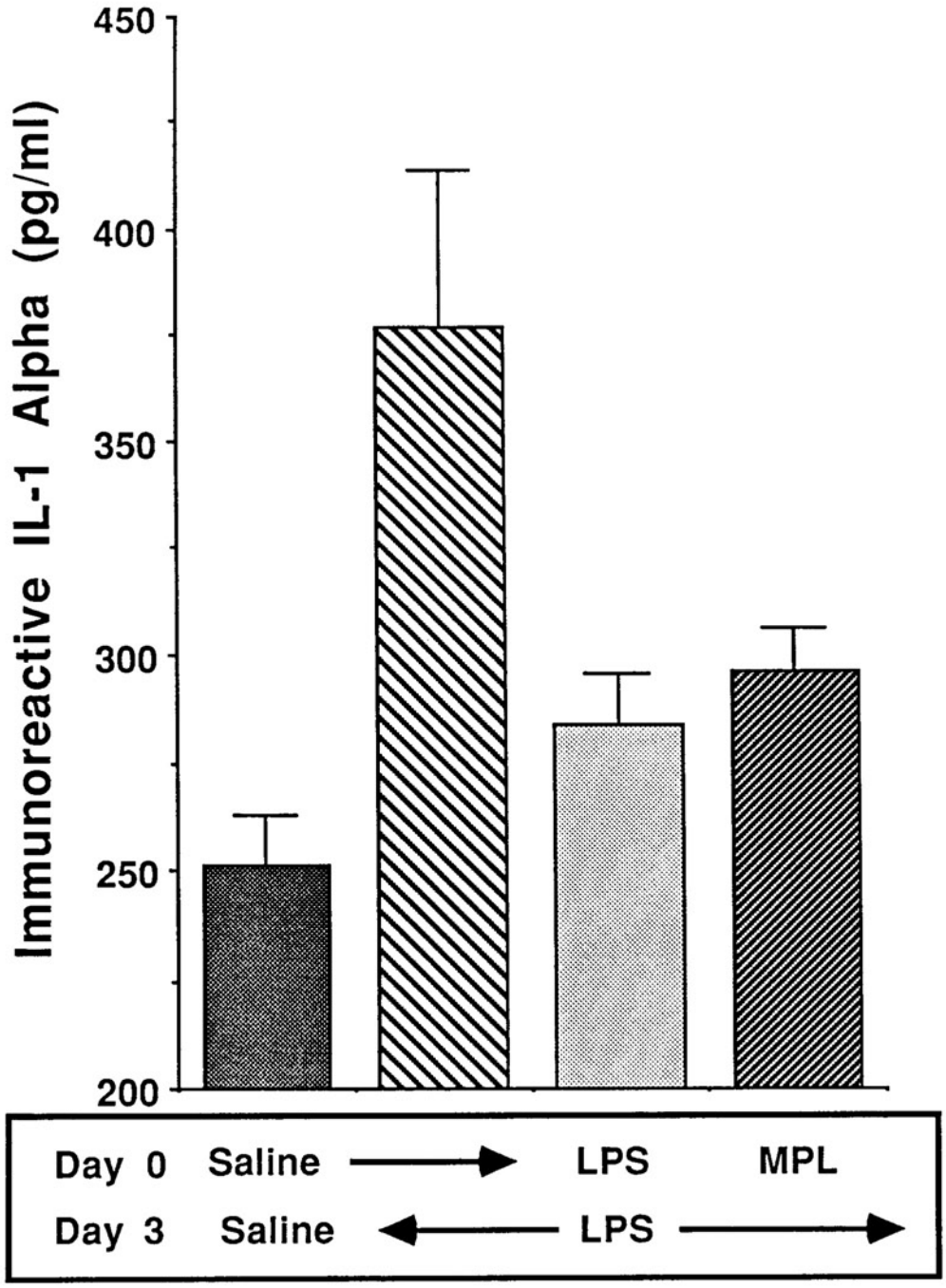
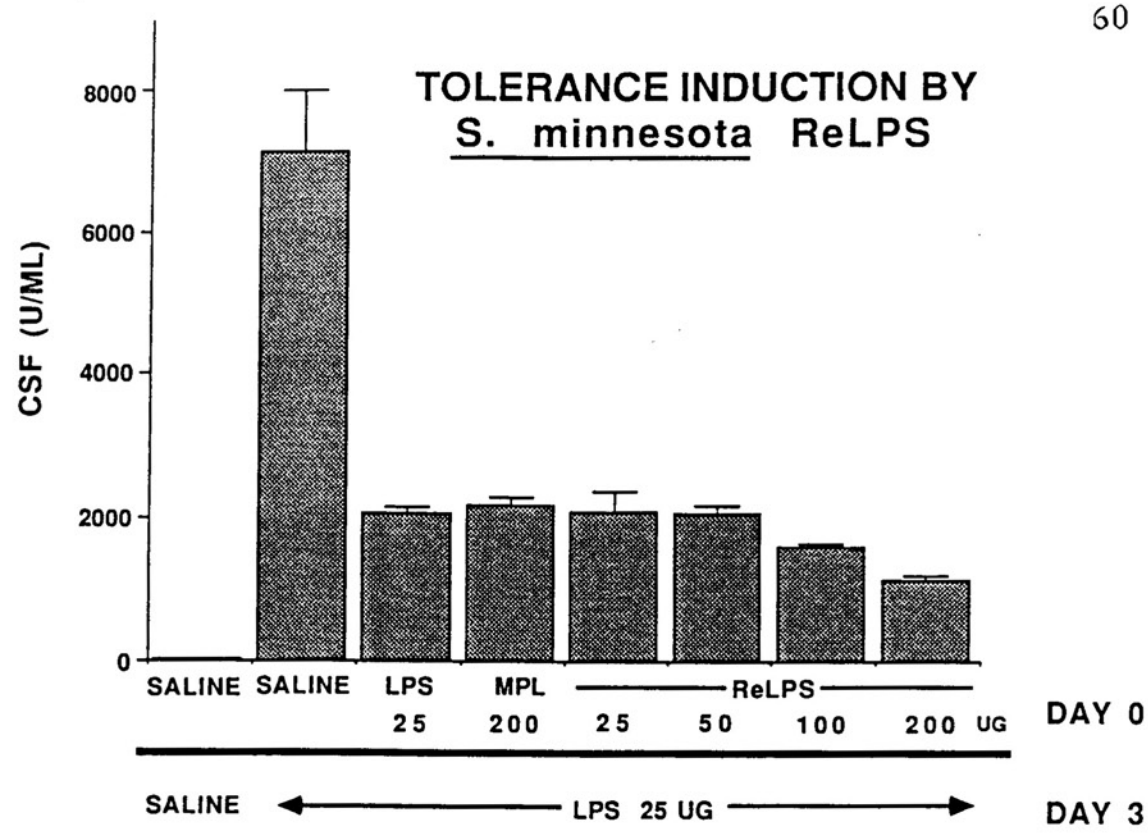
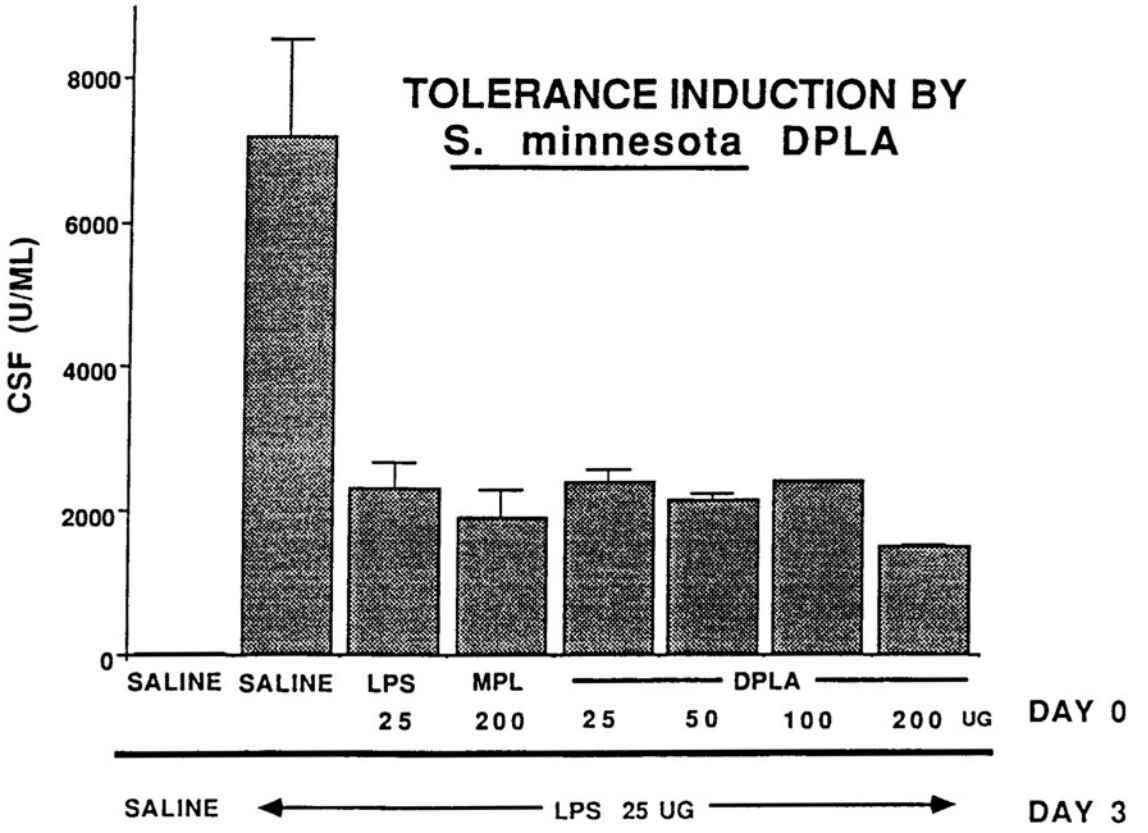

Immunoreactive IL-1 Alpha (pg/ml)
450
400
350
300
250
200
Day 0 Saline
LPS
MPL
Day 3 Saline
LPS
Treatment

Figure 8. Tolerance induction by ReLPS and DPLA as assessed by CSF production. Mice (3-4 per treatment group per experiment) were injected on Day 0 (D0) with Saline (SAL), LPS (25 μg), MPL (200 μg), ReLPS (25 μg, 50 μg, 100 μg, or 200 μg), or DPLA (25 μg, 50 μg, 100 μg, or 200 μg). On Day 3 (D3), one group of saline pretreated mice was reinjected with saline and the others were "challenged" with LPS (25 μg). Six hours after the D3 injection, mice were bled and sera were assayed for CSF activity (U/ml) as described in the Materials and Methods. Treatments are indicated on the X-axis. Results represent the arithmetic means +/- the standard error of the mean for 2 separate experiments.


TOLERANCE INDUCTION BY
S. minnesota ReLPS
CSF (U/ML)
8000
6000
4000
2000
0
SALINE
SALINE
LPS
25
MPL
200
ReLPS
25
50
100
200 UG
DAY 0
SALINE
LPS 25 UG
DAY 3


TOLERANCE INDUCTION BY
S. minnesota DPLA
CSF (U/ML)
8000
6000
4000
2000
0
SALINE
SALINE
LPS
25
MPL
200
DPLA
25
50
100
200 UG
DAY 0
SALINE
LPS 25 UG
DAY 3

200 μg *S. minnesota* MPL, or 200 μg synthetic *E. coli* MPL (Table II).

Induction of Cytokines Upon Initial Injection of LPS *versus* MPL. Since both LPS and MPL induced early endotoxin tolerance, and LPS-induced toxicity was cytokine-mediated, one way to assess the relative capacity of MPL *versus* LPS was to compare the cytokine-inducing ability of both at doses that evoked equivalent tolerance. Therefore, experiments were performed to compare directly levels of cytokines induced by the initial injection of a tolerance-inducing dose of each.

When CSF levels were compared, there were no significant differences between the levels induced by equivalent tolerance-inducing doses of LPS and MPL (Figure 9). Although a tendency was noted for the MPL-induced CSF to decrease after four hours, the difference between LPS- and MPL-induced CSF at 6 hours was not statistically significant. In contrast, when IFN (Table III), TNF (Table IV), and IL-6 (Figure 10) activities were compared after initial injection, MPL elicited significantly less of each cytokine than did the equivalent tolerance-inducing dose of LPS. Maximal IFN, TNF and IL-6 activities induced by MPL were approximately 5-10-fold less than that elicited by LPS. IL-6 production peaked between two and four hours after LPS or MPL injection. As was observed for TNF and IFN, peak IL-6 activity induced by LPS was more than ten-fold greater than that induced by injection of MPL in each of three experiments.

As was observed for IFN, TNF, and IL-6, the induction of circulating IL-1α by 200 μg of MPL was also less than that induced by 25 μg of LPS (Figure 11). It is important to note that the ELISA assay used to measure the presence of IL-1 in serum detects only the IL-1α species, and not IL-1 β. IL-1α has been demonstrated to be the species which is predominantly cell-associated,

Table II

INDUCTION OF EARLY ENDOTOXIN TOLERANCE BY SYNTHETIC *E. COLI* MPL

TREATMENT		CSF ACTIVITY (U/ml)*
Day 0	Day 3	
Saline	Saline	<35 ± 10
Saline	LPS	3025 ± 176
LPS (25 μg)	LPS	1890 ± 386**
Salmonella MPL (200 μg)	LPS	1810 ± 150**
E. coli MPL (200 μg)	LPS	2150 ± 130**
E. coli MPL (100 μg)	LPS	2355 ± 317
E. coli MPL (25 μg)	LPS	3175 ± 251

*Arithmetic mean ± SEM

**Significantly different from Day 0 Saline/Day 3 LPS, $p < 0.05$

Figure 9. CSF activity induced by single injection of LPS (25 μg) or MPL (200 μg). Mice (3-8 per treatment group per experiment) were injected with saline, LPS (25 μg) or MPL (200 μg) at time zero. At one, two, four, and six hours after injection, mice were bled and sera were assayed for CSF activity (U/ml) as described in the Materials and Methods. Numbers in parentheses are the number of separate experiments for the indicated time point. The results represent the arithmetic mean +/- the standard error of the mean. No significant differences between LPS- and MPL- injected groups were observed as assessed by unpaired Student's *t* test.

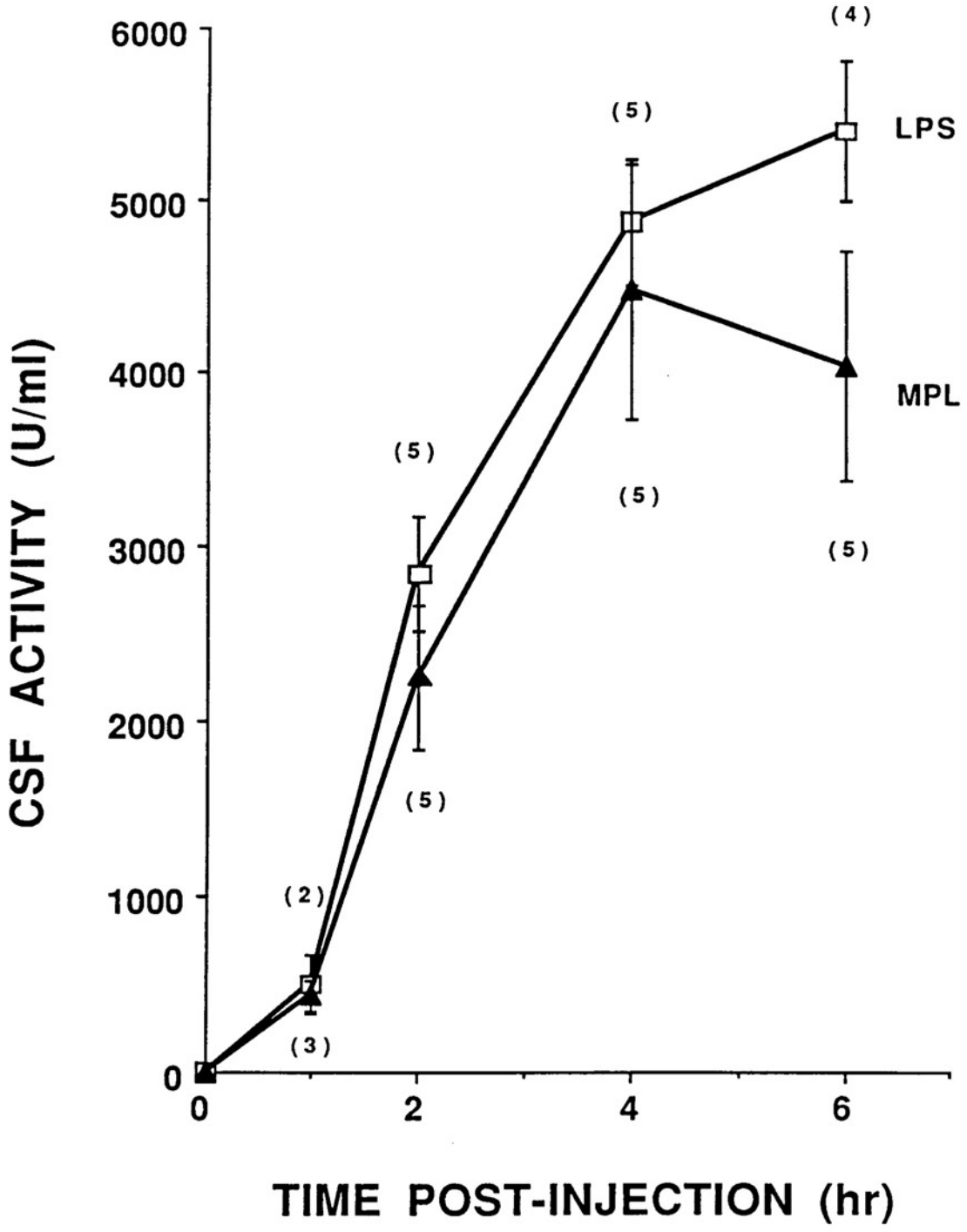

6000
5000
4000
3000
2000
1000
0
0
2
4
6
CSF ACTIVITY (U/ml)
TIME POST-INJECTION (hr)
LPS
MPL
(4)
(5)
(5)
(5)
(5)
(5)
(2)
(3)

Table III.

Induction of Interferon Activity by Injection of LPS *versus* MPL[a]

Time Post-Injection (hr)	IFN Activity (U/ml)	
Saline Only	6 (4 - 8)[b]	
	LPS (25 μg)	MPL (200 μg)
1	21 (8-51)	6 (2-25)
2	370 (241-569)	35 (16-76)
4	232 (138-390)	33 (9-120)
6	161 (88-294)	71 (52-100)

[a]Mice (3 - 7 per treatment group per experiment) were injected with LPS (25 μg) or MPL (200 μg). At the indicated times after injection, mice were bled and the sera assayed for IFN as described in the Materials and Methods.

[b]The values presented represent the geometric means of 4 to 10 separate experiments. The figures in parentheses are the 95% confidence limits for these values.

Table IV.

Induction of Tumor Necrosis Factor Activity by Injection of LPS *versus* MPL

Time Post-Injection (hr)	TNF Activity (U/ml)	
Saline Only	130 (82-207)[b]	
	LPS (25 μg)	MPL (200 μg)
1	9,330 (466-18,676)	761 (318-1,819)
2	2,463 (1,161-5,224)	1,512 (848-2,695)
4	143 (77-266)	113 (56-226)
6	108 (59-195)	113 (56-226)

[a]Mice (3-7 per treatment group per experiment) were injected with LPS (25 μg) or MPL (200 μg). At the indicated times after injection, mice were bled and the sera were assayed for TNF as described in the Materials and Methods.

[b]The values presented represent the geometric means of 4 to 12 separate experiments. The figures in parentheses are the 95% confidence limits for these values.

Figure 10. IL-6 activity produced by single injection of LPS (25 μg) or MPL (200 μg). Mice (3-8 per treatment group per experiment) were injected with Saline, LPS (25 μg), or MPL (200 μg) at time zero. At the indicated times after injection, mice were bled, and sera were assayed for IL-6 activity (U/ml) as described in the Materials and Methods. Open circles, closed triangles, and closed squares indicate the results for three separate experiments.

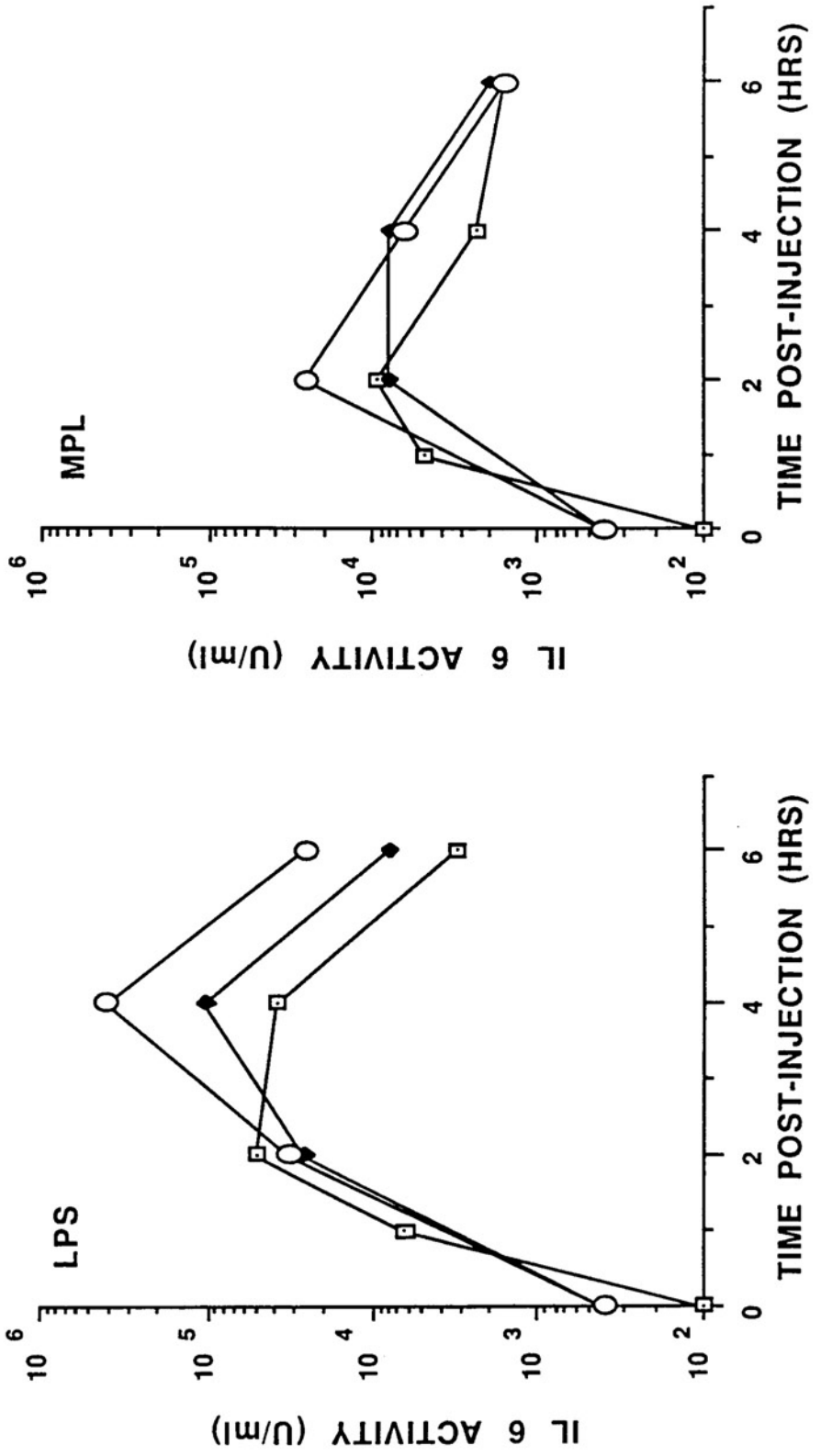
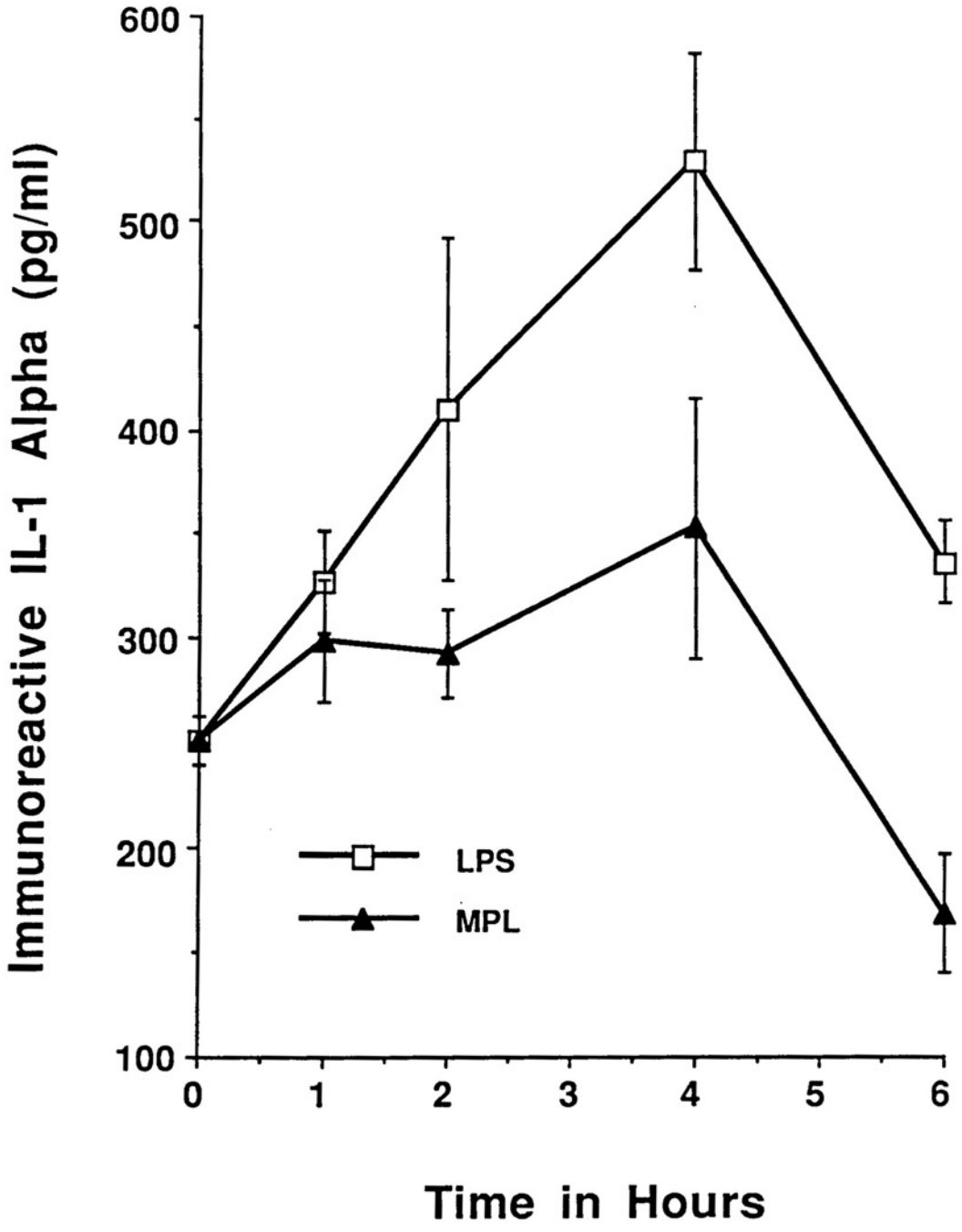

MPL
LPS
IL 6 ACTIVITY (U/ml)
TIME POST-INJECTION (HRS)
0
2
4
6
10^2
10^3
10^4
10^5
10^6

Figure 11. IL-1α protein produced by a single injection of LPS (25 μg) or MPL (200 μg). Mice (3 per treatment group per experiment) were injected with Saline, LPS (25 μg), or MPL (200 μg) at time zero. At one, two, four, and six hours after injection mice were bled, and sera were assayed for IL-1α levels as described in the Materials and Methods. IL-1α concentration is indicated in pg/ml. Time in hours post-injection is indicated on the X-axis. Results shown are the arithmetic means +/- standard error of the mean of individual mouse samples derived from a representative experiment.


Immunoreactive IL-1 Alpha (pg/ml)
600
500
400
300
200
100
0
1
2
3
4
5
6
LPS
MPL
Time in Hours

whereas IL-1β is generally the predominant secreted form (Hooper, 1990). This measurement of immunoreactive protein, therefore, may not reflect the true levels of circulating IL-1 bioactivity .

Thus, when initially exposed to equivalent tolerance-inducing doses of LPS and MPL, the level of one cytokine, CSF, is of comparable magnitude. However, the induction of circulating IFN, TNF, IL-6, and IL-1α are significantly less in mice which have been injected with MPL.

INHIBITORS OF LPS-INDUCED CYTOKINE ACTIVITY

Interleukin 1 was among the first cytokines to be associated with LPS-induced manifestations . IL-1, either alone or in combination with other cytokines, has been demonstrated to induce many of the same physiological responses that are observed after administration of LPS. Among these are fever, hypoglycemia, induction of other cytokines (as well as classical "late" acute phase reactants), resistance to lethal irradiation, increased non-specific resistance to infection, shock, and death (reviewed in Vogel and Hogan, 1990). These effects may be mediated secondarily by other cytokines or acute phase proteins induced by LPS and/or IL-1. However, the direct demonstration that IL-1 indeed serves as an intermediate in LPS-induced responses can only be demonstrated by blocking the specific LPS-mediated effect with an IL-1 antagonist. For instance, both LPS and recombinant IL-1 (rIL-1) have been shown to induce CSF activity *in vivo* (Vogel *et al.*, 1987). However, this does not prove that IL-1 functions as an intermediate in this LPS-induced phenomenon. Similarly, injection of a sublethal dose of LPS has been shown to diminish LPS-responsiveness three days later (referred to as "early endotoxin tolerance") and this phenomenon can be simulated by injection of animals with a combined regimen of recombinant IL-1α and TNFα (Vogel *et al.*, 1987). However, the finding that these two cytokines synergize to induce a state akin to "early endotoxin tolerance" does not prove that either cytokine is a direct intermediate in this LPS-mediated phenomenon.

Like IL-1, TNF has been shown to mediate many LPS-induced effects as well (Dinarello *et al.*, 1986; Bauss, *et al.*, 1987; reviewed in Vogel, 1992). When polyclonal anti-TNF antibody became available, it was shown that

administration of the anti-cytokine antibody could protect against lethal exposure to LPS (Beutler *et al.*, 1985). In 1990, Vogel and Havell showed that a polyclonal recombinant anti-murine TNF antibody could reduce LPS-induced CSF, and that TNF, therefore, was a direct intermediate in the induction of LPS-induced CSF activity. However, they also showed that administration of anti-TNF antibody had no effect on LPS-induced interferon or corticosterone levels, nor did it ameliorate LPS-induced hypoglycemia, indicating that TNF was not a mediator of these LPS-induced effects (Vogel and Havell, 1990).

A recently described inhibitor of IL-1 is a 22 kDa IL-1 receptor antagonist (IL-1ra) that occurs naturally as a human, macrophage-derived protein with an unglycosylated and two alternate glycosylated states (Arend *et al.*, 1990; Hannum *et al.*, 1990). The cDNA of this protein has now been cloned and the recombinant protein produced in an *E. coli* expression system (Eisenberg *et al.*, 1990). There exist two receptors for IL-1: one which predominates on fibroblasts and T cells ("Type 1") and one which predominates on B cells and macrophages ("Type 2") (Chizzonite *et al.*, 1989; Hannum *et al.*, 1990). This protein binds to both human IL-1 receptor types (Dr. J. Sims, Immunex Corp., personal communication) and to the high binding capacity (e.g., the "Type 1") murine IL-1 receptor (Bomsztyk *et al.*, 1989; Hannum *et al.*, 1990), and has been shown to inhibit IL-1-induced prostaglandin E_2 and collagenase secretion from synovial cells *in vitro* (Arend *et al.*, 1990).

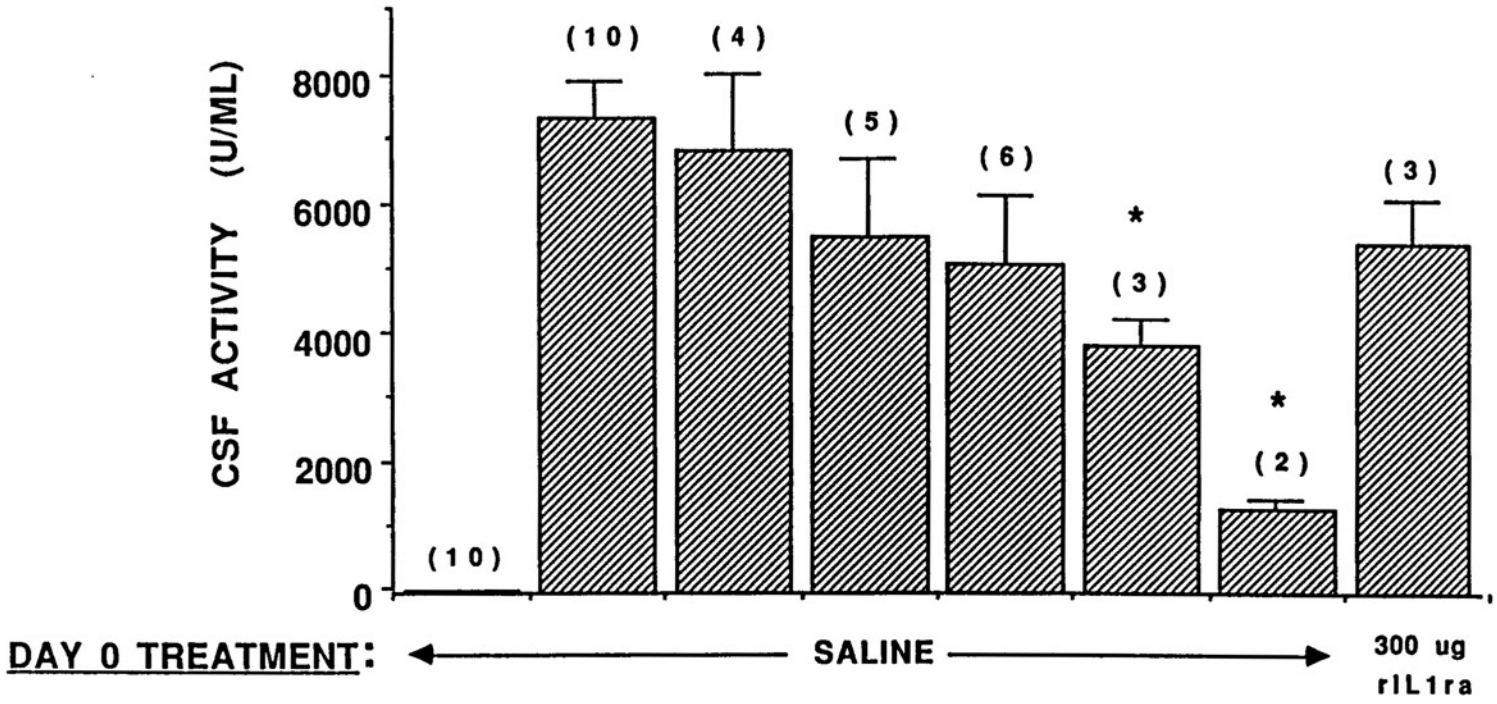
In this study, rIL-1ra was utilized to test whether: (i) IL-1 is an intermediate in the induction of LPS-induced colony stimulating factor (CSF); (ii) IL-1 contributes to LPS-induced hypoglycemia; and, (iii) IL-1 is an intermediate in the induction of "early endotoxin tolerance" induced by LPS and MPL. The combined effects of polyclonal anti-TNF antibody and/or rIL-1ra on

LPS-induced tolerance were also measured, as assessed by its effects on LPS-induced CSF.

The effect of rIL-1ra on LPS-induced CSF activity and hypoglycemia *in vivo*. The effect of rIL-1ra upon LPS-induced CSF activity was first examined (Figure 12). At the highest concentration tested (600 μg), rIL-1ra inhibited LPS-induced CSF by >80% ($p = 0.002$). Lower doses of rIL-1ra blocked production of CSF in a dose-dependent fashion. The rIL-1ra exhibited no CSF-inducing capacity of its own when injected, and in no case was it toxic, even at a dose of 600 μg/mouse (33 mg/kg). A lesser degree of inhibition of LPS-induced CSF could be observed even if the antagonist (300 μg rIL-1ra per mouse) were administered 3 days prior to LPS injection. Although not statistically significant, this "carry over" effect of the rIL-1ra is approximately equivalent to the blocking capacity of 15 μg rIL-1ra administered simultaneously with LPS (Figure 12). The rIL-1ra-mediated decrease in CSF activity was not accompanied by any apparent decrease in colony size. The rIL-1ra had no stimulatory or inhibitory effects on myeloid stem cell proliferation in the CSF bone marrow colony assay.

A dose of 300 μg rIL-1ra also reversed LPS-induced hypoglycemia significantly (Table V). Upon injection, a 25 μg dose of LPS induces a predictable level of hypoglycemia, depressing the serum glucose level to approximately 54% of the saline control group (Compare Group A to Group B). Although injection of rIL-1ra alone had no affect on the serum glucose by itself (Group C), a 300 μg dose was able to reverse LPS-induced hypoglycemia significantly (Compare Group D to Group B). Recombinant IL-1ra, partially, but significantly, reversed hypoglycemia, even when the 300 μg dose was given three days prior to LPS administration (Compare Groups B and E). The extent of reversal of the hypoglycemia was comparable to that observed in mice that have been rendered tolerant to the administration of LPS (Group F).

Figure 12. The effect of rIL-1ra on LPS-induced CSF. Saline or 300 μg rIL-1ra was injected into C57BL/6J mice (4 - 5 mice per treatment group per experiment) on Day 0. Saline, 25 μg *E.coli* K235 LPS and/or rIL-1ra were injected on Day 3. Serum samples for CSF determination were obtained 6 hours after injection on Day 3. Numbers in parentheses indicate the number of separate experiments for each treatment group. Asterisks indicate group means which were found to be significantly different by Student's *t* test ($p < 0.05$) from mice which had received saline on Day 0 and LPS on Day 3.


CSF ACTIVITY (U/ML)
8000
6000
4000
2000
0
(10)
(10)
(4)
(5)
(6)
*
(3)
*
(2)
(3)
DAY 0 TREATMENT:
SALINE
300 ug rIL1ra
DAY 3 TREATMENT:
SALINE
LPS
LPS + 1.5 ug rIL1ra
LPS + 15 ug rIL1ra
LPS + 150 ug rIL1ra
LPS + 300 ug rIL1ra
LPS + 600 ug rIL1ra
LPS

Legends to Table V.

[a]Mice were injected on Day 0 with either saline, rIL-1ra (300 μg), and/or LPS (25 μg) and then challenged three days later (Day 3) with saline, LPS (25 μg), rIL-1ra (300 μg), or LPS plus rIL-1ra, as indicated. Mice were bled 6 hours after the Day 3 injection and serum glucose levels were measured as described in the Materials and Methods.

[b]Results represent the arithmetic means of serum glucose levels measured on pooled serum samples (4-5 mice per treatment per group per experiment) from the indicated number [n] of individual experiments. Differences were assessed by unpaired Student's *t* test, and p values for specific comparisons are reported in parentheses.

Table V

Effect of rIL-1ra on LPS-Induced Hypoglycemia

Treatment Group (Day0/Day3)[a]		Serum Glucose Levels +/- sem (mg/dl) [n]	(p)[b]
A.	Saline/Saline	105.0 ± 3.1	[12]
B.	Saline/LPS (25 μg)	56.7 ± 3.4	[14] ($p < 0.001$, A vs. B)
C.	Saline/rIL-1ra (300 μg)	100.9 ± 5.9	[6]
D.	Saline/LPS + rIL-1ra (300 μg)	79.1 ± 4.6	[3] ($p = 0.011$, B vs. D)
E.	rIL-1ra (300 μg)/LPS	79.9 ± 3.3	[4] ($p = 0.003$, B vs. E)
F.	LPS/LPS	80.4 ± 7.1	[10] ($p = 0.003$, B vs. F)

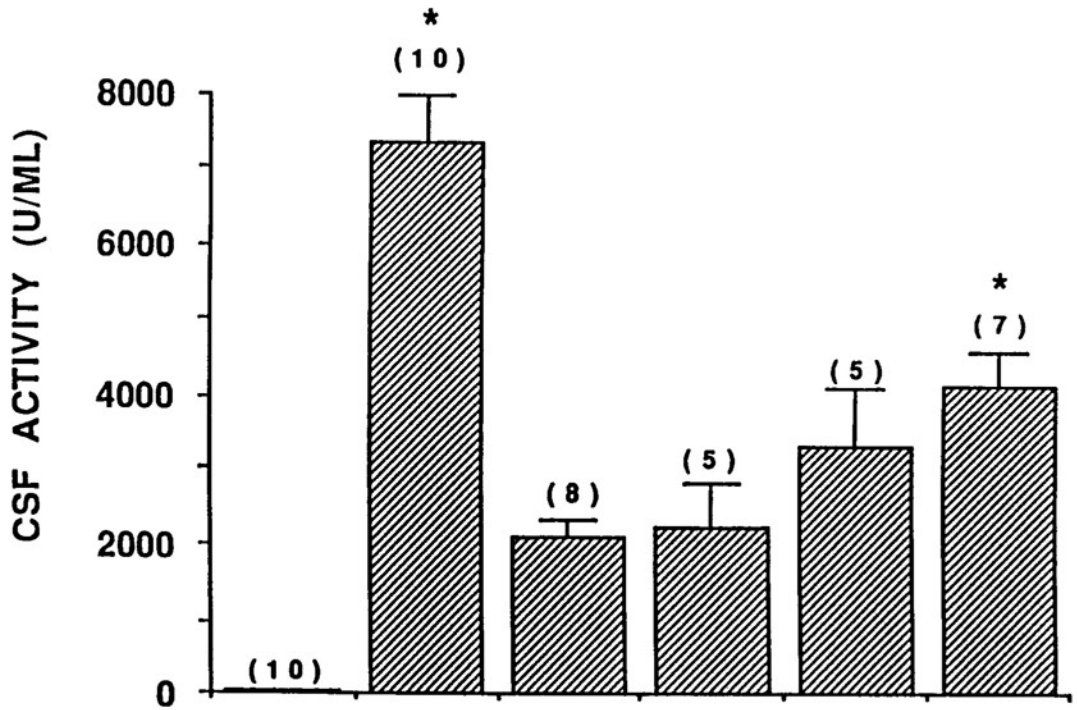
Legend on facing page

Effect of rIL-1ra on induction of early endotoxin tolerance *in vivo.*

Treatment of mice with rIL-1ra also partially reversed induction of early endotoxin tolerance by LPS. In the absence of the rIL-1ra, exposure to LPS on Day 0 resulted in a markedly diminished capacity to respond to LPS 3 days later to produce CSF (Figure 13; $p < 0.001$), as previously reported (Madonna and Vogel, 1985; Williams *et al.*, 1983). The capacity of the rIL-1ra to reverse induction of early endotoxin tolerance was also dose-dependent. When 300 μg rIL-1ra was administered simultaneously with the initial (Day 0) LPS injection, CSF production in response to the challenge (Day 3) injection of LPS was increased over tolerant state levels by almost 100% ($p = 0.001$) (Figure 13). Recombinant IL-1ra also reversed MPL-induced tolerance as evidenced by a return of CSF production after a 25 μg challenge injection of LPS on Day 3 (Figure 14). When injected simultaneously with a Day 0 pretreatment of 200 μg MPL, 300 μg rIL-1ra reversed tolerance to the same level as it did when given in combination with an equivalent tolerance-inducing dose of LPS (25 μg).

Effect of anti-TNF antibody on induction of early endotoxin tolerance *in vivo*. Previous studies demonstrated that 5,000 neutralizing units of anti-TNF antibody blocked LPS-induced CSF by ~50% (Vogel and Havell, 1990), similar to the action of 300 μg rIL-1ra (Figure 12). In preliminary experiments, these findings were confirmed and an *in vivo* titration of anti-TNF antibody was carried out prior to using this reagent to assess the role of TNF in the induction of early endotoxin tolerance by LPS. We sought to identify a concentration of anti-TNF antibody that did not block LPS-induced CSF 3 days after its initial administration, to preclude a "carry over" effect. Figure 15 illustrates that 10,000 neutralizing units of anti-TNF antibody, an amount in excess of that required to block CSF induction by LPS when administered simultaneously (Vogel and

Figure 13. The effect of rIL-1ra on LPS-induced early endotoxin tolerance. Saline, 25 μg of *E. coli* K235 LPS, or 25 μg LPS plus the indicated dose of rIL-1ra was injected into C57BL/6J mice (4 - 5 mice per treatment group per experiment) on Day 0. On Day 3, mice were re-injected with saline or "challenged" with 25 μg LPS . CSF activity was determined on sera collected 6 hours later. Numbers in parentheses indicate the number of separate experiments for each treatment group. Asterisks indicate group means which were found to be significantly different ($p < 0.05$) by Student's *t* test from mice which had received LPS on Days 0 and 3.



DAY 0 TREATMENT:	SALINE	SALINE	LPS	LPS + 15 ug rIL1ra	LPS + 150 ug rIL1ra	LPS + 300 ug rIL1ra
DAY 3 TREATMENT:	SALINE	LPS	LPS	LPS	LPS	LPS

Figure 14. The effect of rIL-1ra on MPL-induced early endotoxin tolerance. Saline, 25 μg *E. coli* K235 LPS, or 200 μg MPL and/or rIL-1ra (300 μg) were injected into C57BL/6J mice (3 mice per treatment group per experiment) on Day 0. On Day 3, mice were reinjected with saline or "challenged" with 25 μg LPS. CSF activity was determined on sera collected 6 hours later as described in the Materials and Methods. The results represent the arithmetic mean of replicate samples +/- the standard deviation of the mean. Asterisks indicate group means which were found to be significantly different ($p<0.05$) by Student's *t* test from mice which had received LPS on Days 0 and 3.

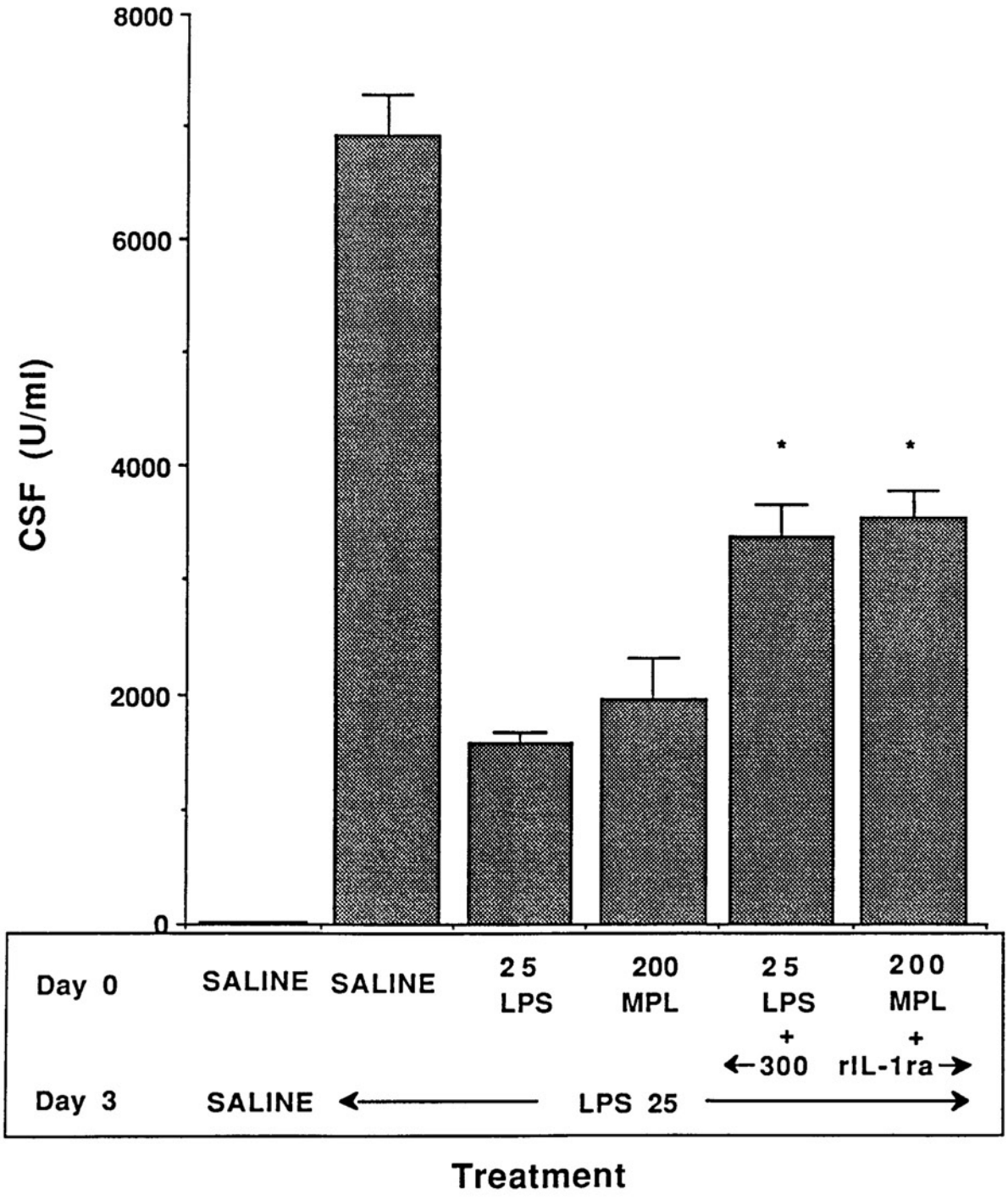
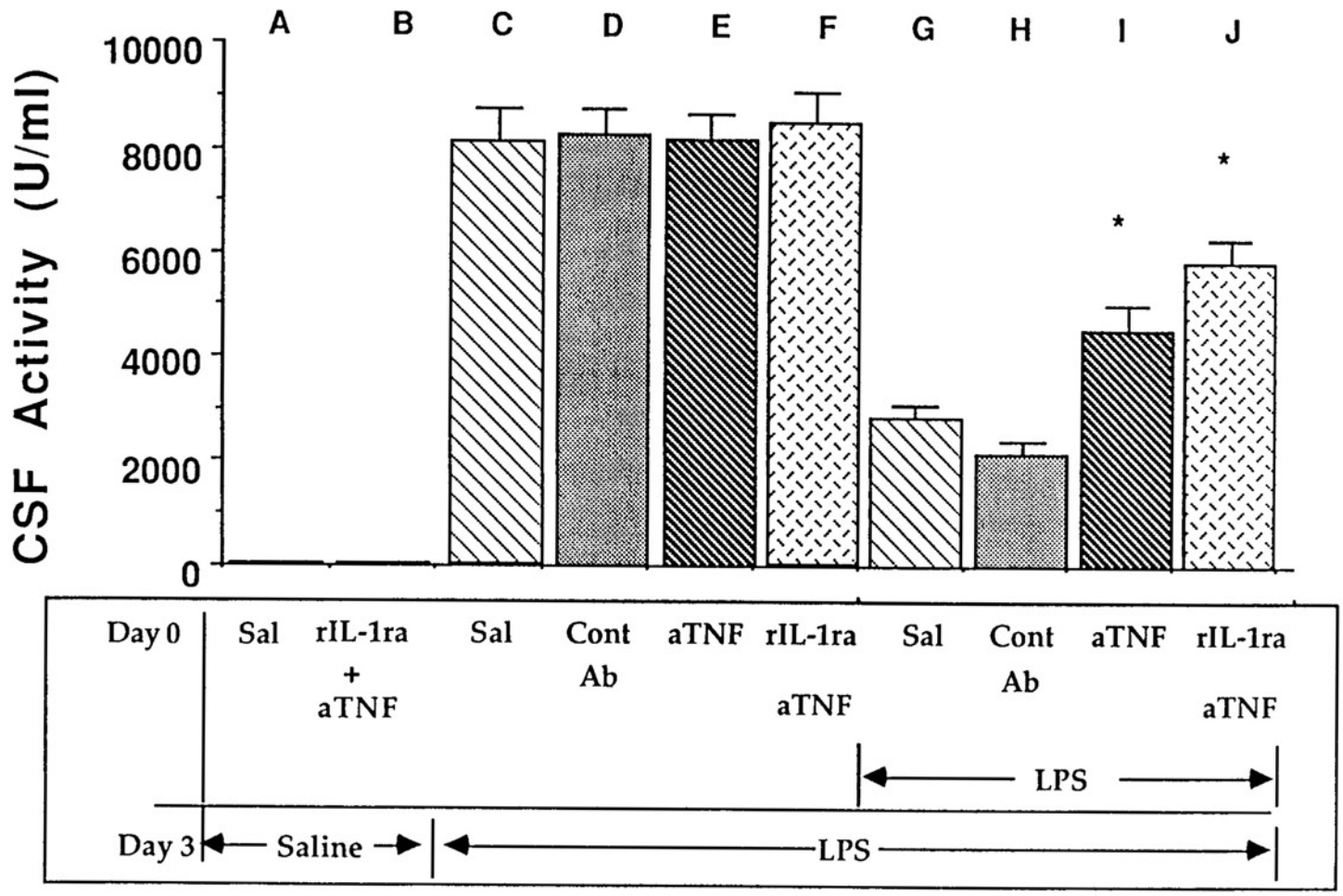

8000
6000
4000
2000
0
CSF (U/ml)
*
*
Day 0
SALINE
SALINE
25 LPS
200 MPL
25 LPS + 300
200 MPL + rIL-1ra
Day 3
SALINE
LPS 25
Treatment

Figure 15. The effect of anti-TNF antibody on LPS-induced early endotoxin tolerance. C57BL/6J mice (3-4 mice per treatment group per experiment) were injected on Day 0 with saline, anti-TNF antibody (aTNF; 10,000 n.u.), control IgG antibody (Cont Ab), or a combination of aTNF (10,000 n.u.) and rIL-1ra (300 μg), as indicated. Certain groups of these mice (Groups G through J) were also administered 25 μg LPS. Three days later, mice were challenged with saline (Groups A and B), or LPS (Groups C-J), and CSF activity was determined on sera collected 6 hours later, as described in the Materials and Methods. The results represent the arithmetic mean $\pm$ standard error of the mean of replicate samples from 2 separate experiments.


A
B
C
D
E
F
G
H
I
J
10000
8000
6000
4000
2000
0
CSF Activity (U/ml)
*
*
Day 0
Sal
rIL-1ra + aTNF
Sal
Cont Ab
aTNF
rIL-1ra aTNF
Sal
Cont Ab
aTNF
rIL-1ra aTNF
LPS
Day 3
Saline
LPS
Treatment

Havell, 1990), failed to reverse LPS-induced CSF 3 days later (Compare columns C and E). Moreover, when 10,000 neutralizing units of anti-TNF antibody was administered in combination with 300 μg rIL-1ra, no CSF was induced by the inhibitors alone (Column B), nor was the level of CSF elicited after a Day 3 challenge injection of 25 μg LPS diminished (Column F). However, when 10,000 neutralizing units of anti-TNF antibody was given on Day 0, without or with 300 μg rIL-1ra, along with a tolerance-inducing dose of 25 μg LPS (Columns I and J), the level of tolerance induced on Day 3 was mitigated significantly. The reversal of tolerance by the presence of the two cytokine antagonists was not greater than that achieved by administration of anti-TNFα alone. Incomplete reversal of tolerance may be due to the inability of either or both antagonist(s) to reach the appropriate compartment, since both were administered intraperitoneally. Tissue penetration may not be sufficient to distribute the antagonists to the appropriate body area to stop cytokine secretion totally.

MACROPHAGE-LPS INTERACTIONS

Macrophage -LPS interactions have been studied extensively *in vitro* and can be divided into four major categories. First, LPS can engage in non-polar interaction with macrophage cell membranes as the macrophage phagocytoses LPS, which results in the non-specific intercalation of the fatty acid residues of LPS directly into the macrophage cell lipid bilayer. This results in the dissolution of cellular substructures such as the mitochondrial, golgi, endoplasmic reticulum, and nuclear membranes (Kang *et al.*, 1990). Second, LPS has been shown to interact directly with a number of cell membrane-associated binding proteins. These proteins include: the ~80 kDa lipid A receptor protein first found on splenocytes (Lei and Morrison, 1988a, 1988b; Roeder *et al.*, 1989); the 47 kDa inner core-specific receptor found on hepatocytes (Parent, 1989, 1990); the 18 kDa membrane-associated protein in 70/3Z pre-B cells (Tobias and Kirkland, 1988); the 95 kDa protein of RAW 264.7 macrophage-like cells that may be an integrin β chain (Hampton *et al.*, 1988); and, scavenger receptors for a variety of altered proteins that also have been shown to bind and clear lipid IV_A from the circulation and to render it less toxic by dephosphorylation (Golenbock *et al.*, 1990; Hampton *et al.*, 1991). The presence of all of these proteins on normal macrophage cell surfaces has yet to be proven. However, interaction of LPS with at least one of these cellular receptors, i.e., the ~80 kDa receptor, appears to activate macrophage effector functions such as cytokine secretion and tumoricidal activity. This conclusion was based on the capacity of a monoclonal antibody specific for the 80 kDa LPS-binding protein to stimulate LPS-responsive macrophages, but not C3H/HeJ (Lps^d) macrophages (Chen *et al.*, 1990; Morrison *et al.*, 1990; Bright *et al.*, 1990).

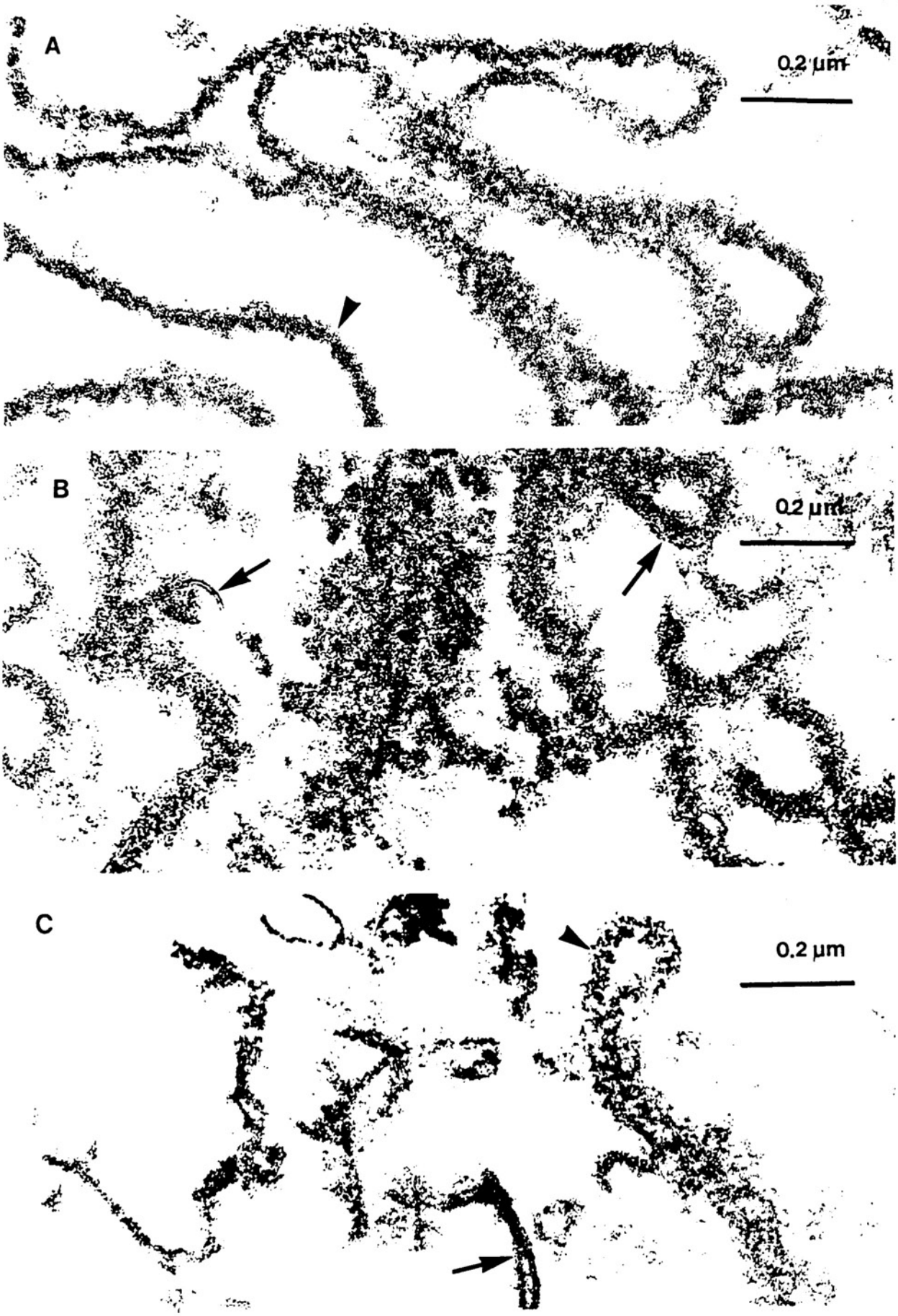
Third, LPS has been shown to interact with hepatocyte-derived acute phase reactant proteins (Tobias and Ulevitch, 1983; Tobias *et al.*, 1985; 1986; 1988; Wright *et al.*, 1989) or LPS-induced antibody to form complexes (Johns *et al.*, 1983; Chia *et al.*, 1989). These complexes are either removed from the circulation via Fc receptor-mediated phagocytosis or they act to increase the non-specific phagocytic removal of LPS from the circulation. They can activate the complement system, (Kuhlman *et al.*, 1989; Schweinle, 1989), or promote recognition of LPS-protein complexes by macrophage cell surface receptors such as CD14. CD14 acts as a receptor for the serum LPS Binding Protein (LBP)-LPS complex (Tobias *et al.*, 1985;1986;1988; Schumann *et al.*, 1990; Wright *et al.*, 1989; 1990). Engagement of the CD14 receptor by LPS-LBP complex has been estimated to be approximately 100-fold more efficient than the interaction of LPS directly with macrophages. It has also been shown in murine 70/3Z cells transfected with the human CD14 gene that LPS-LBP interaction with CD14 results in a 10,000-fold increase in surface IgM expression (Lee *et al.*, 1992).

The fourth mechanism by which LPS interacts with macrophages is intracellular. Several intracellular constituents have been shown to interact with LPS and may have a bearing on the ultimate ability of the macrophage to respond to LPS effectively. Hampton *et al.* (1988) have found a 31 kDa LPS binding protein in the RAW 264.7 macrophage cell line that is histone-like and is localized primarily in the nucleus, raising questions concerning the direct effect of LPS-protein complexes on DNA structural integrity and transcriptional processes. Also, Munford and colleagues (Hall and Munford, 1983; Munford and Hall, 1985) have isolated an enzyme acyloxyacyl hydrolase (AOAH) from a human promyelocytic cell line that is active in the deacylation of LPS by macrophages. AOAH may play a significant role in the detoxification of LPS.

Freudenberg *et al.* (1986,1987) have determined that the macrophage plays a central role not only in LPS responsiveness, but also in the induction of tolerance. Therefore, it was desirable to gain insight into how LPS and MPL differ with respect to their ability to interact with macrophages. The effects of LPS *versus* MPL on macrophages *in vitro* were studied utilizing four distinct approaches: (1) Differences between the ability of LPS and MPL to interact with red blood cell membranes were delineated by electron microscopy; (2) The competition of LPS *versus* MPL for binding to a putative LPS receptor was examined using a polyclonal monospecific anti-lipid A receptor antibody; (3) LPS and MPL binding to peritoneal exudate macrophages was compared by use of *R. sphaeroides* DPLA, which has been shown to be a specific lipid A antagonist (Takayama *et al.*, 1989; Qureshi *et al.*, 1991; Kirkland *et al.*, 1991); and (4) The induction of AOAH activity by LPS *versus* MPL, and the possible contribution of AOAH-mediated deacylation and detoxification of LPS in endotoxin tolerance, were explored.

Comparison of LPS and MPL interaction with cell membranes using electron mcroscopy. The first method chosen to study the physical interaction of LPS and MPL with cell membranes and to delineate potential differences between micelle formation of both under physiologic conditions, was electron microscopy. When equivalent concentrations of LPS and MPL were allowed to interact at room temperature for 1 hour with SRBC, which has been used as a model of non-specific interaction under physiologic conditions. The SRBC were then gently lysed and immobilized (see Materials and Methods), and LPS appeared to form smaller micelles that were more intimately associated with and integrated into the cell membranes (Figure 16 A). MPL formed longer, laminated bilayer and triplet aggregates, and showed end-on-attachment that led to the formation of asterisk-like and V-shaped structures (Figure 16 B).

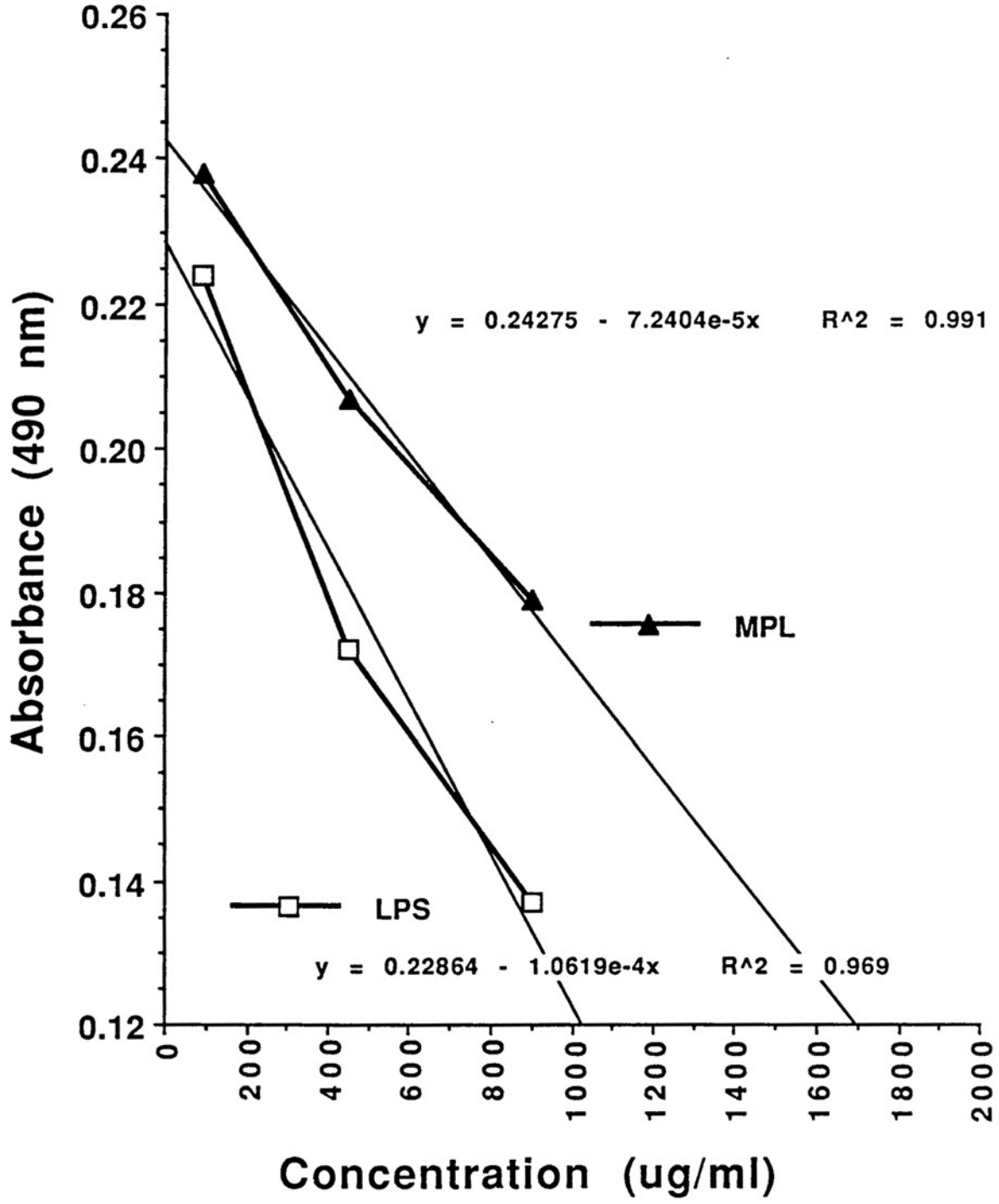
Figure 16. Electron micrographs of the interaction of LPS and MPL with hypotonically lysed sheep red blood cell stroma (Figure 16 A). LPS (Figure 16 B), and MPL (Figure 16 C) were compared for interaction with sheep red blood cell stroma. LPS and MPL at 1 mg/ml were added to fresh SRBCs and were incubated at 37° C for 1 hour. Cells were then hypotonically lysed, fixed with glutaraldahyde, stained with 2% osmium tetroxide, and treated as described in the Materials and Methods prior to sectioning. Individual sections were stained with uranyl acetate. Photomicrographs were taken at a magnification of 40,000 X (Figure 16 A) and 20,000 X (Figures 16 B and C) and magnified 2.5 and 4.56 times, respectively, during printing for a total magnification of 100,000 X (Figure 16 A) and 91,200 X (Figures 16 B and C), respectively. Bar on each photo indicates 0.2 μm for that photo. Small arrowheads (A) and (C) indicate hypotonically lysed SRBC stroma sufficiently disrupted to obliterate the unit membrane. Large arrows indicate (B) LPS micelles in contact with and integrated into membrane material, and (C) MPL aggregates showing end-on attachment to disrupted membrane material. .


A
0.2 µm
B
0.2 µm
C
0.2 µm

Although LPS and MPL micelles in solutions that are prepared and solubilized identically in triethylamine appear initially to form micelles of the same size and appearance (Ribi *et al.*, 1986), the electron micrographs in Figure 16 suggest that they interact quite differently with cell membranes. The electron microscopy results suggest that the higher dose of MPL that is required *in vivo* to induce cytokines and endotoxin tolerance may be due to formation of larger micelles by MPL, perhaps formed due to the removal of the phosphoryl group at the 1 position. Removal of the large charged group results in a compound that is more hydrophobic (Rietschel *et al.*, 1987). Surrounding water under physiologic conditions may cause stabilization of micellar structures, and by reinforcing the required interaction of MPL's hydrophobic fatty acid groups, lead to an effective sequestration of a larger fraction of MPL's fatty acids from direct interaction with the membrane.

Competitive displacement LPS and MPL by an anti-LPS receptor protein antibody. Morrison and his colleagues have identified an ~80 kDa lipid A-binding protein on the surface of murine and human lymphoid cells (Lei *et al.*, 1988). They prepared a polyclonal antibody to this receptor which was shown by immunoprecipitation and Western blot analysis to be specific for the 80 kDa antigen. This polyclonal, monospecific rabbit anti-lipid A receptor antibody (Rα5D3) (Chen *et al.*, 1992) was used to demonstrate the capacity of LPS *versus* MPL to block the binding of the antibody to the macrophages, as described in the Materials and Methods. When concentrations of LPS and MPL from 90 to 1000 μg/ml were used to compete with a fixed concentration of anti-receptor antibody for binding to the macrophage ~80 KDa surface protein, a higher concentration of MPL *versus* LPS was required to displace an equivalent amount of antibody. The concentrations at which LPS and MPL were each able to inhibit the binding of

Figure 17. Competitive inhibition of Rα5D3 antibody binding to peritoneal exudate macrophages by LPS and MPL. Peritoneal exudate macrophages were treated with the indicated concentrations of LPS and MPL as described in the Materials and Methods. At the end of 60 minutes incubation at 37° C, a fixed concentration of Rα5D3 antibody was added and incubation was continued at 4° C for 45 minutes to re-establish equilibrium. Antibody binding was detected by a standard horseradish peroxidase ELISA, and absorbance was read at 490 nm. Results are the arithmetic mean of 9 separate experiments, corrected for binding of an irrelevant antibody for each LPS and MPL concentration. Comparative slopes of the resultant lines were calculated by regression analysis of the curve. Equations of the ideal lines are indicated for both LPS and MPL with the coefficient of correlation (R^2) of the experimental data to the ideal line. The concentration at which LPS and MPL inhibited 50% of the Rα5D3 antibody binding was calculated by substituting the value of 50% of the absorbance of the primary antibody alone (0.0925) for (x) in each equation and solving for the value of (y).

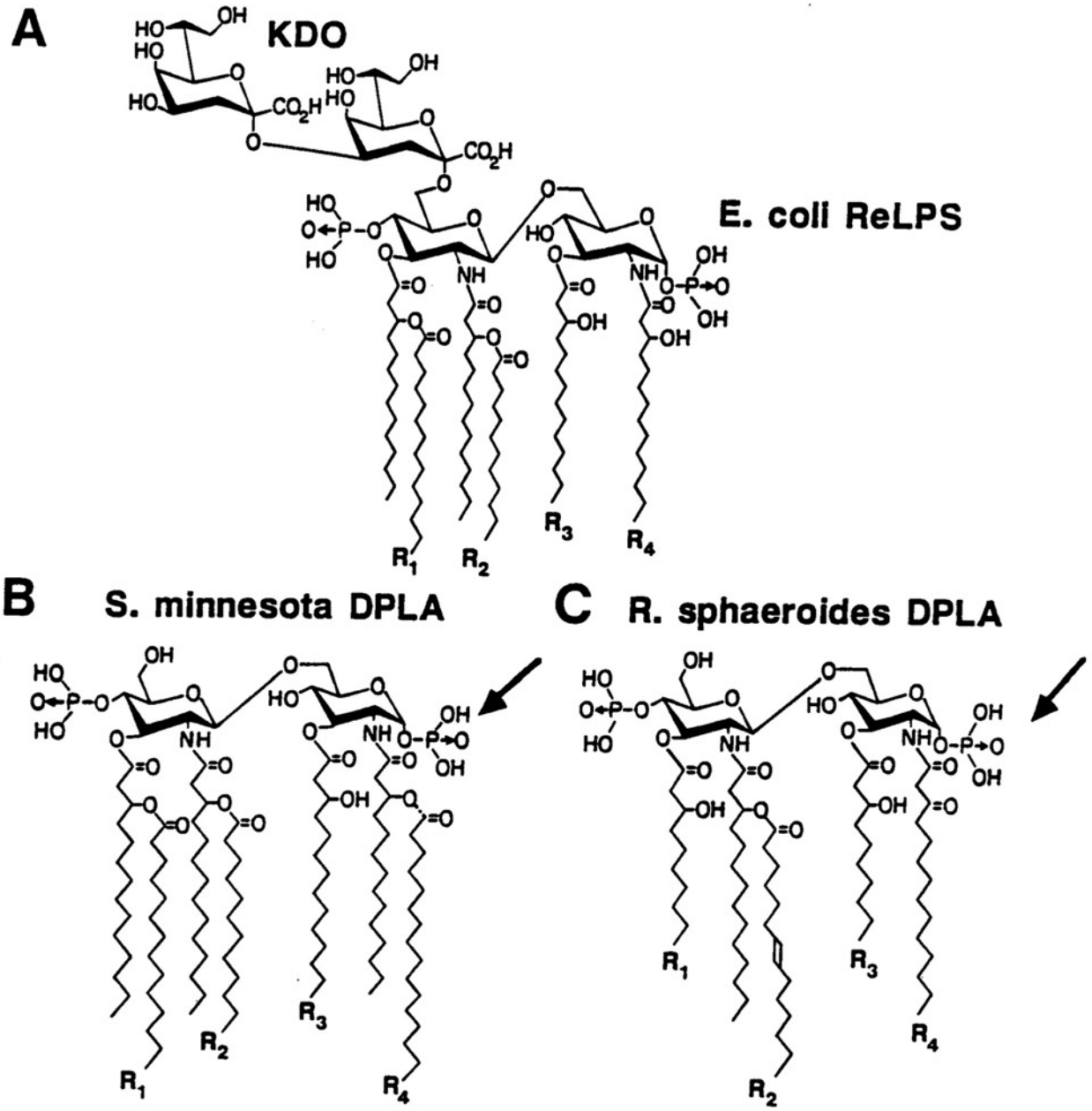

0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
Absorbance (490 nm)
y = 0.24275 - 7.2404e-5x R^2 = 0.991
MPL
LPS
y = 0.22864 - 1.0619e-4x R^2 = 0.969
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Concentration (ug/ml)

antibody by 50% were 1283 μg/ml and 2075 μg/ml, respectively (Figure 17). This data, coupled with the greater slope of the line of inhibition for LPS *versus* MPL, suggests that LPS had a somewhat higher binding capacity for the ~80 kDa receptor than did MPL, although quantification of binding capacity differences was not possible using this method.

Blockade of LPS-induced TNF secretion and the reversal of tolerance *in vitro* by *Rhodopseudomonas sphaeroides* DPLA. The structure and toxicity of LPS, and more specifically, its diphosphorylated acylated glucosamine disaccharide (e.g., lipid A), is species specific. Though inherently heterogeneous, the composition of the LPS of a number of species is sufficiently well-defined to make study of structure *versus* function possible. Careful study of the fatty acid composition and phosphorylation patterns of various lipid A species has shown that the most toxic lipid A is the diphosphoryl hexaacyl variety which occurs naturally in *E. coli* and *Salmonellae* (Brade *et al.*, 1988; Rietschel *et al.*, 1987a; 1987b; Takada and Kotani, 1989, Takayama *et al.*, 1984).

It has been shown that addition of a seventh acyl group, alteration of the pattern or length of the lipid A fatty acids, or dephosphorylation of the lipid A results in a decrease in the bioactivity of the structure for eukaryotic cells (Ribi, 1984; Rietschel *et al.*, 1972, Takayama *et al.*, 1984). The lipid A derived from *Rhodopseudomonas sphaeroides* ATCC 17023 has been studied extensively, since it has been shown to be non-toxic (Strittmatter *et al.*, 1983; Salimath *et al.*, 1983). Its structure has now been completely determined via nuclear magnetic resonance imaging and plasma desorption mass spectrometry (Qureshi *et al.*, 1988, 1991a). The LPS of *R. sphaeroides* contains an unusual pentaacylated lipid A (Figure 18C), which has 3-hydroxydecanoic acids at R1 and R3 rather than the 3-hydroxytetradecanoic acids present in biologically active *E. coli* lipid A . In

Figure 18. Chemical structures of the lipopolysaccharide derivatives used in experiments utilizing *R. sphaeroides* DPLA. Arrows indicate the position of phosphoryl group that is removed from the monophosphoryl derivatives used in these studies.

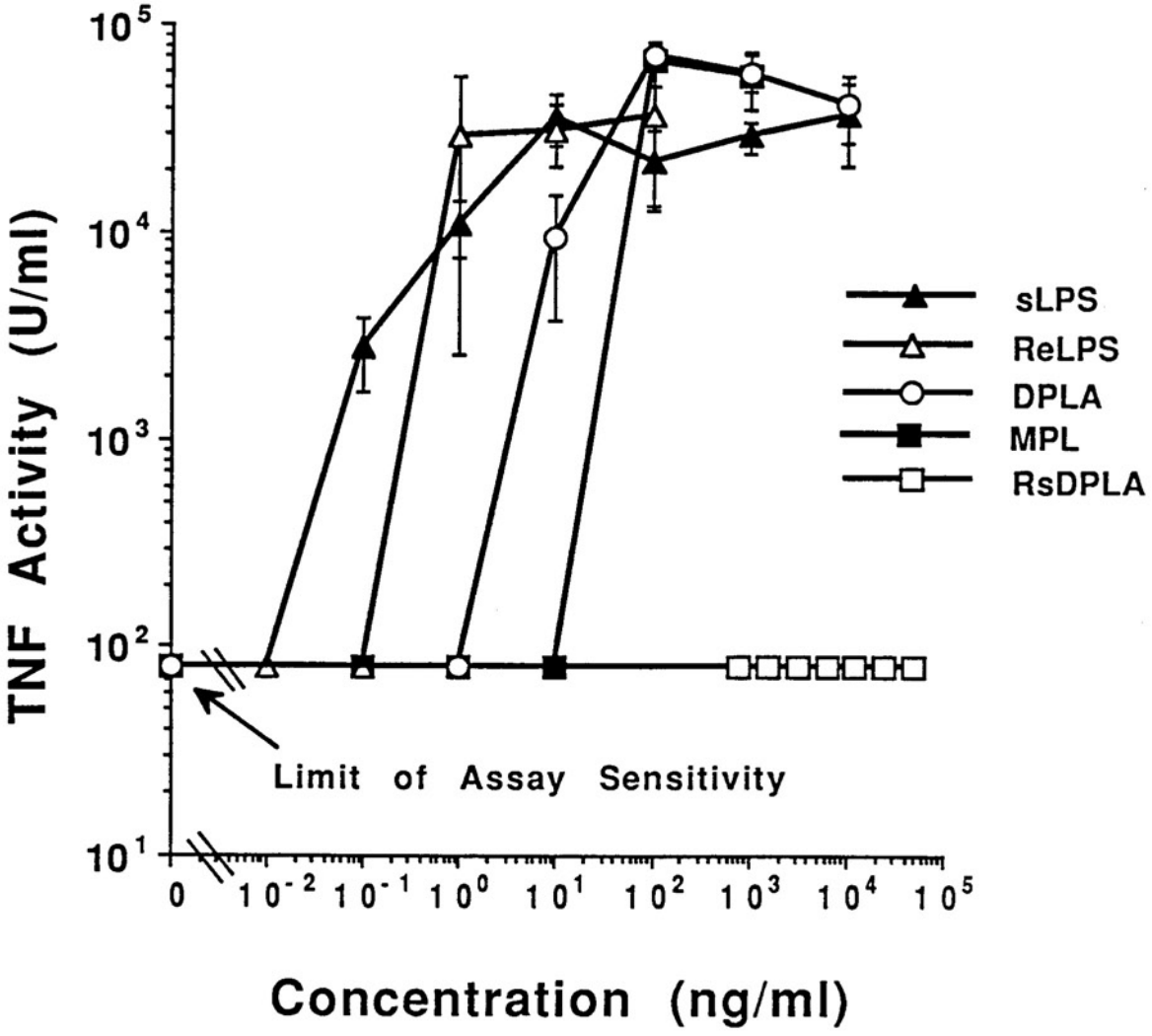

A
KDO
E. coli ReLPS
B
S. minnesota DPLA
C
R. sphaeroides DPLA

addition, it possesses an unsaturated bond in a 14 carbon acyloxyacyl group linked to the 14 carbon R2, and a keto group, instead of a hydroxyl group, on carbon 3 of the C_{14} fatty acid at R4. The diphosphoryl lipid A of *R. sphaeroides* (RsDPLA) has been shown to act as an antagonist to toxic LPS, as it blocks TNF secretion induced by *E. coli* ReLPS (Figure 18 A) from the RAW 264.7 macrophage-like cell line (Takayama *et al.*, 1989), and inhibits LPS-induced activation of a pre-B cell line (70Z/3) (Kirkland *et al.*, 1991). RsDPLA also acts as an endotoxin antagonist for mice *in vivo* and blocks LPS-induced IL-1 secretion from peritoneal exudate macrophages (Qureshi *et al.*, 1991b).

The availability of an inactive lipid A analogue which appears to act by blocking the interaction of LPS or lipid A with its receptor (s) on the macrophage (Golenbock *et al.*, 1991), made it possible to examine the comparative binding of LPS *versus* MPL to macrophages. The capacity of RsDPLA and its monophosphoryl derivative (RsMPLA) to antagonize the binding of LPS and its derivatives (including MPL), was examined *in vitro* in this series of experiments.

Induction of TNF by RsDPLA and bio-active LPS derivatives. RsDPLA (Figure 18 C) is an unusual form of pentaacylated lipid A which has been shown previously (Takayama *et al.*, 1989) to block induction of TNF secretion from the RAW 264.7 macrophage cell line stimulated by an *E. coli*-derived ReLPS (Figure 18 A). These findings are confirmed and extended in Figure 19 to include the smooth hexaacylated LPS of *E. coli* K235 (sLPS), the Re chemotype LPS of *E. coli* D31m4 (ReLPS), *S. minnesota* diphosphoryl lipid A (DPLA; Figure 18 B), and *S. minnesota* monophosphoryl lipid A (MPL). With regard to TNF secretion, sLPS is approximately 10-fold, 100-fold, and 1,000-fold more potent on a weight basis than ReLPS, DPLA, and MPL, respectively. Even at concentrations as high as 50 μg/ml, the RsDPLA failed to induce macrophages to secrete TNF (Figure 19).

Figure 19. TNF induction by RsDPLA *versus* lipid A derivatives and sLPS. Adherent peritoneal exudate macrophages were incubated for 20 hours with media alone or the indicated concentrations of LPS or derivatives. Each data point represents the results of three to eight separate experiments. Error bars are the standard error of the geometric mean.

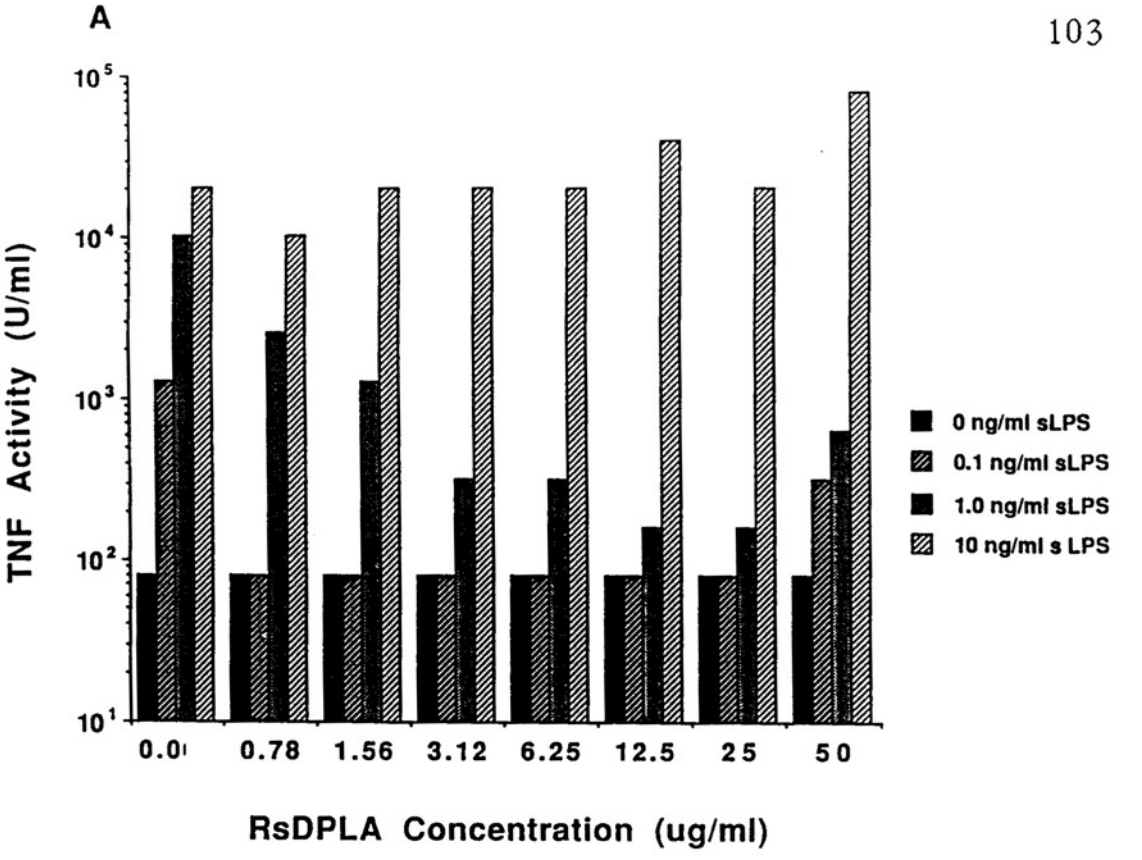

TNF Activity (U/ml)
10^5
10^4
10^3
10^2
10^1
0
10^{-2} 10^{-1} 10^0 10^1 10^2 10^3 10^4 10^5
Concentration (ng/ml)
Limit of Assay Sensitivity
sLPS
ReLPS
DPLA
MPL
RsDPLA

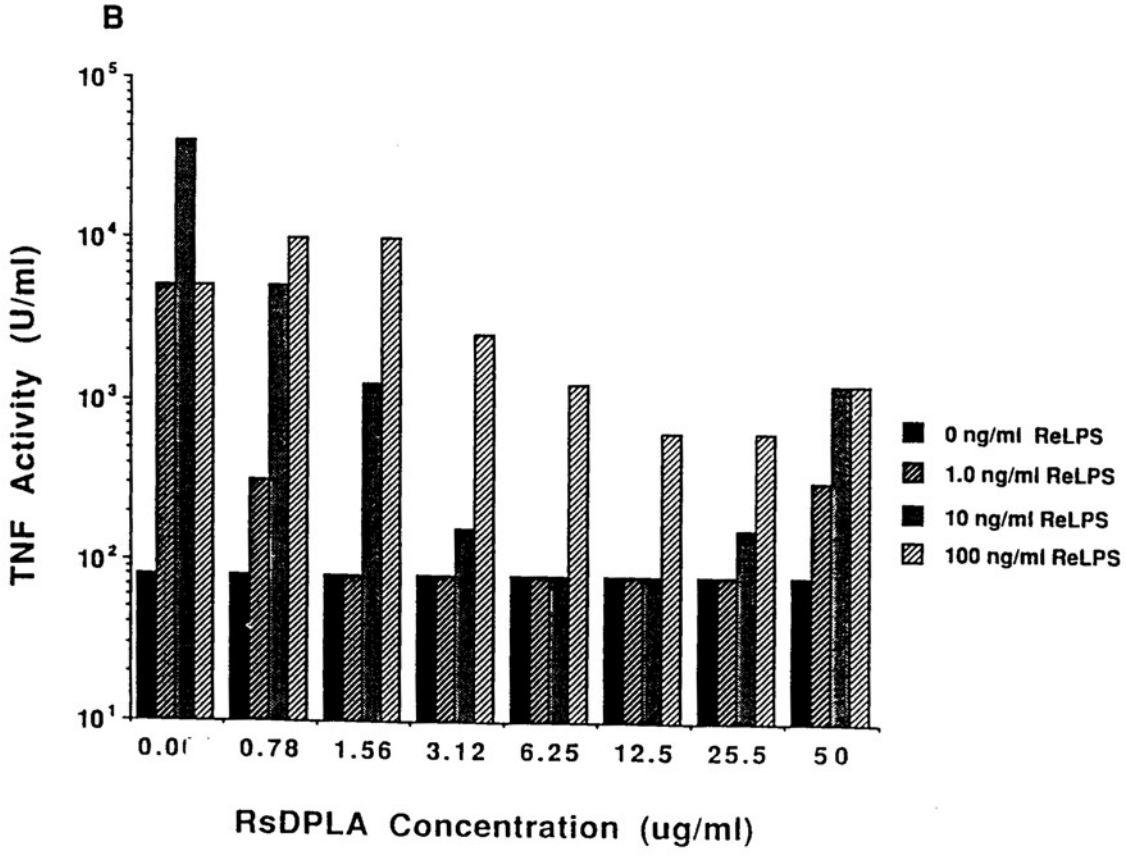
Blocking of LPS-induced TNF secretion by RsDPLA. The ability of RsDPLA to block competitively the secretion of TNF from peritoneal exudate macrophages treated with sLPS, ReLPS, DPLA, and MPL was found to be consistent with the relative capacity of each derivative to induce TNF (Figure 20). RsDPLA blocked TNF secretion induced by 1 ng/ml sLPS (Figure 20 A), 10 ng/ml ReLPS (Figure 20 B), 100 ng/ml DPLA (Figure 20 C), and 1,000 ng/ml MPL (Figure 20 D) equivalently and in a concentration-dependent fashion. Concentrations of sLPS, ReLPS, DPLA, and MPL greater than these were less efficiently blocked by RsDLPA. Again, RsDPLA by itself failed to induce TNF even at 50 μg/ml. The derivative, RsMPLA, also blocked TNF secretion induced by 1 ng sLPS, 10 ng/ml ReLPS, 100 ng/ml DPLA, and 1,000 ng/ml MPL, but much higher concentrations of RsMPLA than RsDPLA were required to block all but the *S. minnesota* MPL derivative (Figure 21).

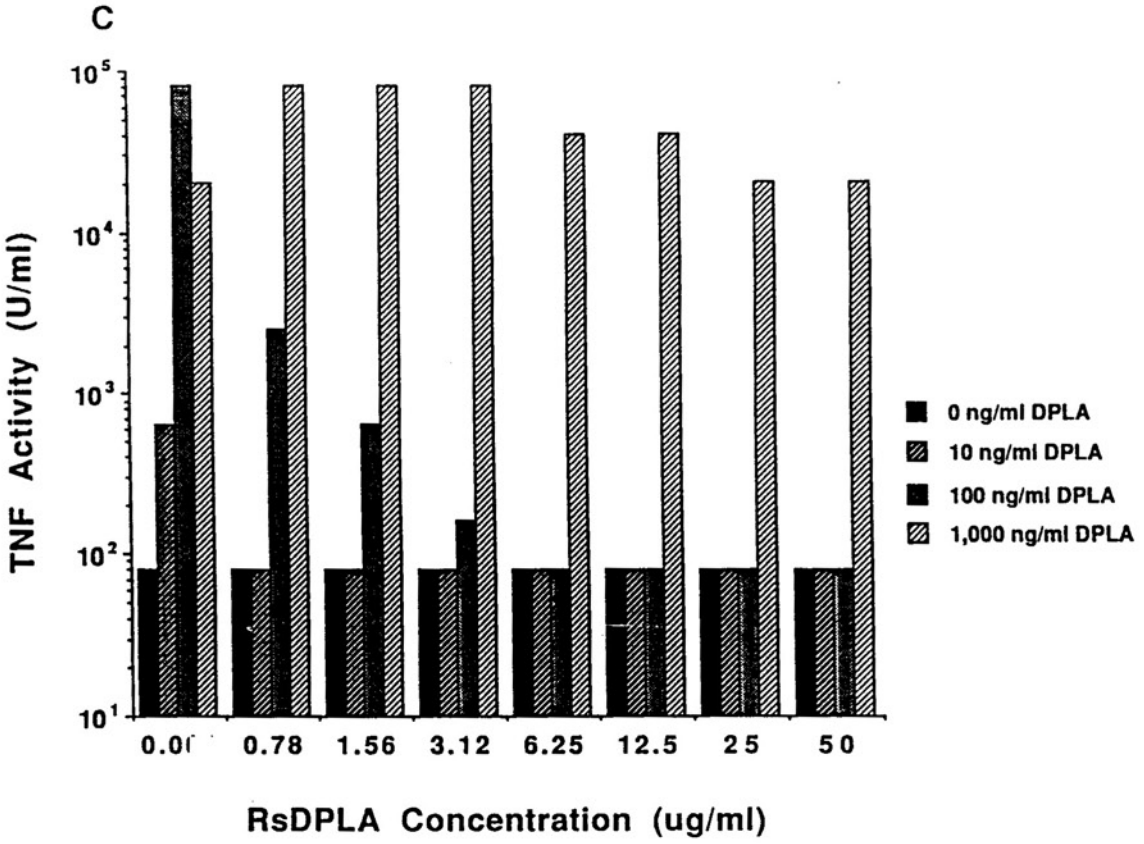
Induction of in vitro tolerance by sLPS, MPL, and RsDPLA. Previous studies have demonstrated that a pre-exposure of the macrophage-like RAW264.7 cell line to sLPS *in vitro* results in the failure of these cells to respond to subsequent stimulation by 10 ng/ml sLPS 19 hours later to produce TNF (Virca *et al.*, 1989). Both sLPS and MPL pretreatment of peritoneal exudate macrophages for 20 hours resulted in a decrease in TNF upon restimulation with 10 ng/ml sLPS (Figure 22). *In vitro* tolerance was induced approximately equivalently by 10 ng/ml sLPS and 100-1,000 ng/ml MPL. Unlike sLPS or MPL, concentrations of RsDPLA as high as 50 μg/ml failed to induce "tolerance" to smooth LPS challenge of 10 ng/ml.

Blocking induction of tolerance with RsDPLA. We next assessed the capacity of RsDPLA to block the induction of *in vitro* tolerance induced by sLPS or MPL. As was seen in Figure 22, in the absence of RsDPLA (0 μg/ml), a 20 hour exposure of macrophages to 10 - 100 ng/ml sLPS (Figure 23 A), or 100-1,000

Figure 20. Comparative blocking of TNF secretion by RsDPLA. Results show the competetive blocking of LPS-stimulated TNF secretion from adherent peritoneal exudate macrophages by RsDPLA. The results are representative data derived from one of 16 separate experiments. Macrophages were pretreated with the indicated concentrations of RsDPLA for 2 hours prior to stimulation for 20 hours with the indicated concentrations of sLPS (Figure 20 A), ReLPS (Figure 20 B), DPLA (Figure 20 C), MPL (Figure 20 D).


A
TNF Activity (U/ml)
10^5
10^4
10^3
10^2
10^1
0.0
0.78
1.56
3.12
6.25
12.5
25
50
RsDPLA Concentration (ug/ml)
0 ng/ml sLPS
0.1 ng/ml sLPS
1.0 ng/ml sLPS
10 ng/ml s LPS


B
TNF Activity (U/ml)
10^5
10^4
10^3
10^2
10^1
0.0
0.78
1.56
3.12
6.25
12.5
25.5
50
RsDPLA Concentration (ug/ml)
0 ng/ml ReLPS
1.0 ng/ml ReLPS
10 ng/ml ReLPS
100 ng/ml ReLPS


C
TNF Activity (U/ml)
10^5
10^4
10^3
10^2
10^1
0.0
0.78
1.56
3.12
6.25
12.5
25
50
RsDPLA Concentration (ug/ml)
0 ng/ml DPLA
10 ng/ml DPLA
100 ng/ml DPLA
1,000 ng/ml DPLA

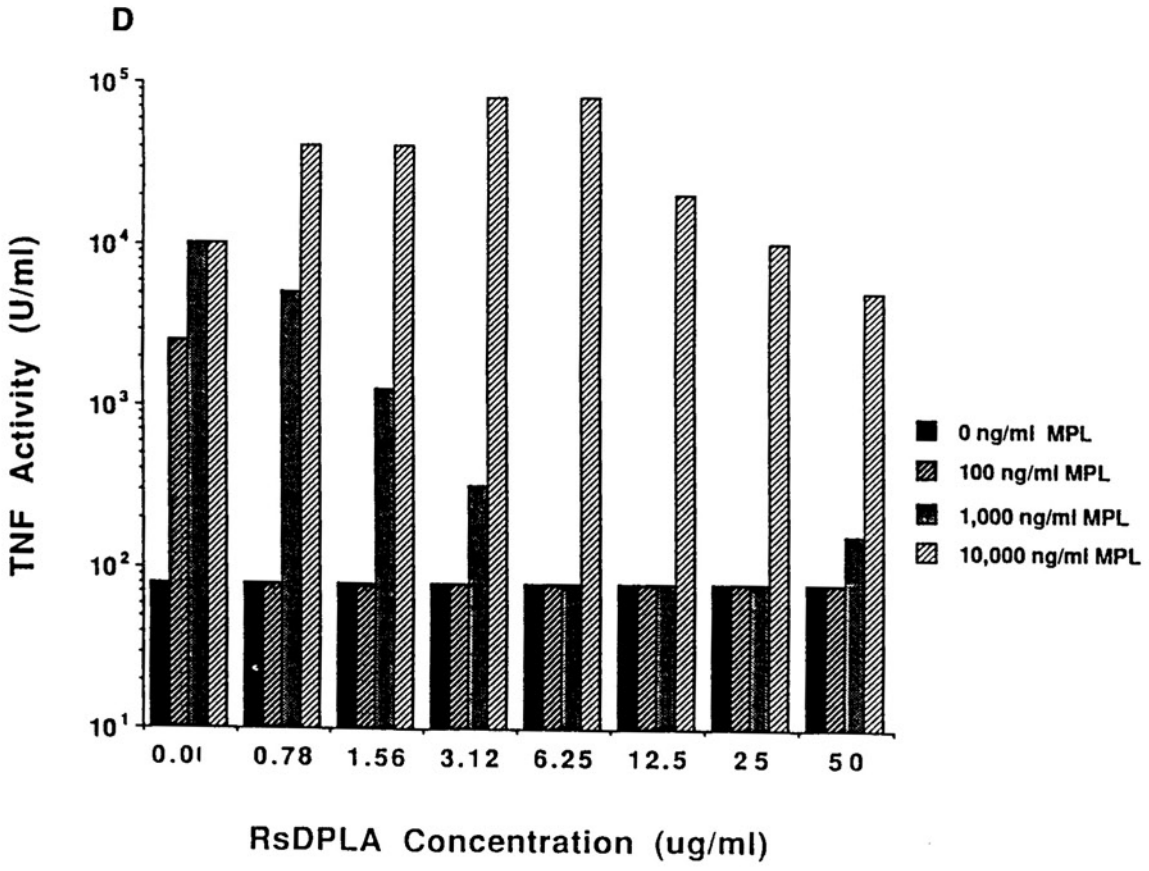
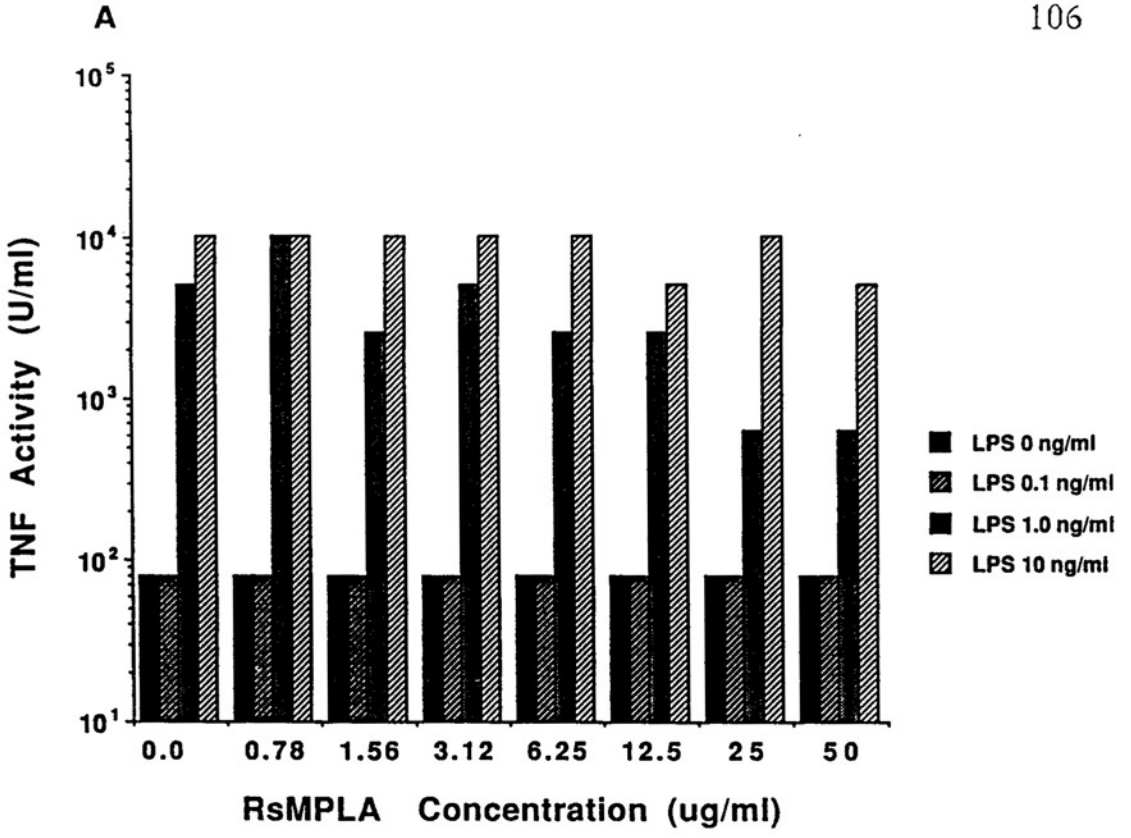
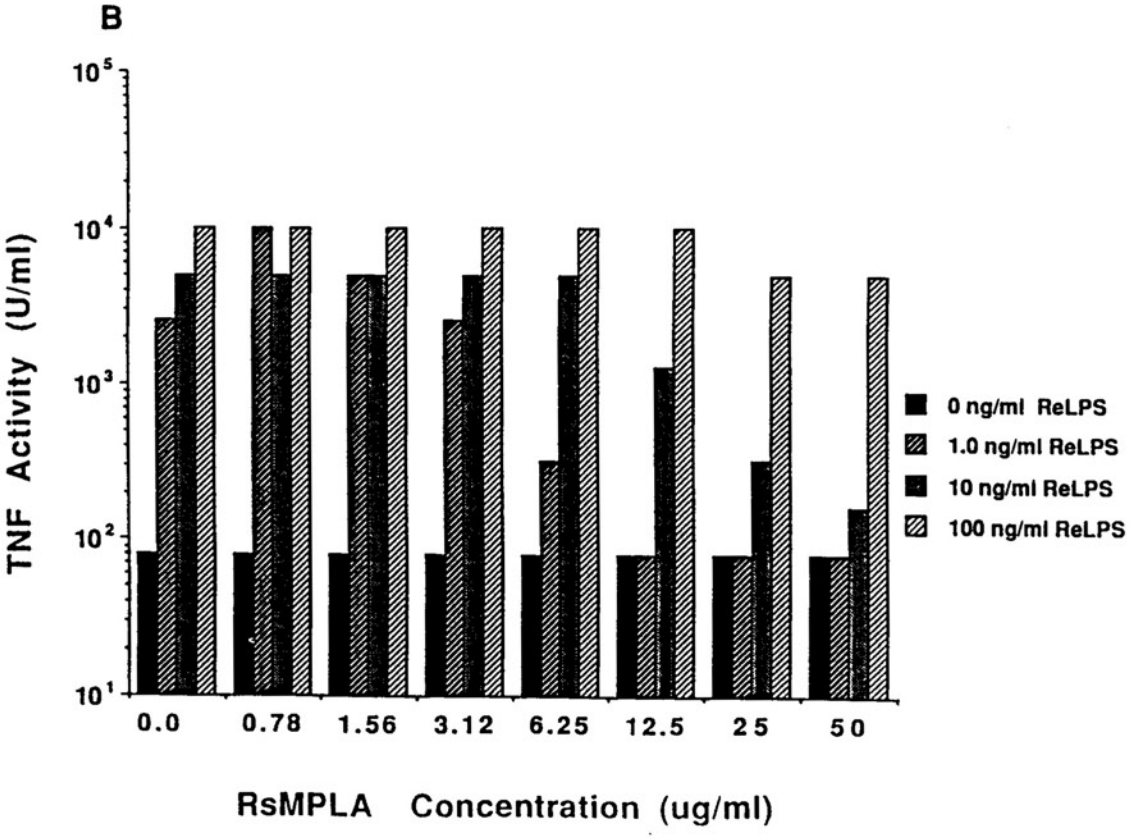
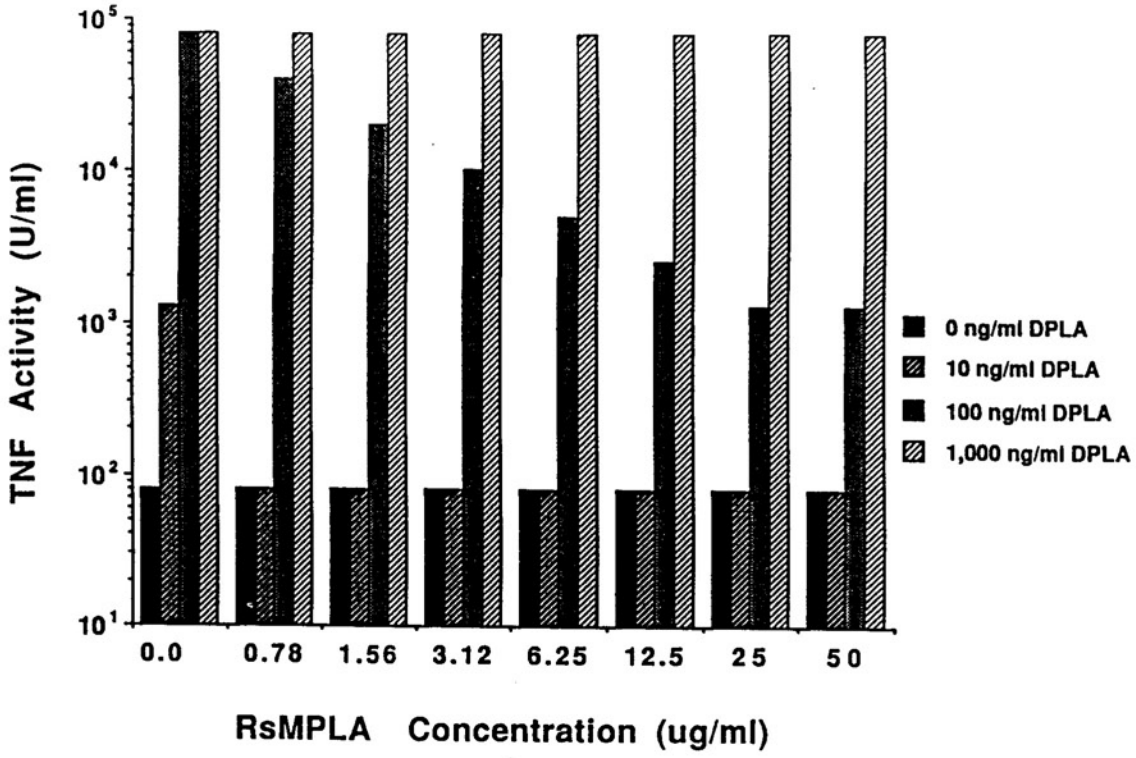

D
TNF Activity (U/ml)
10^5
10^4
10^3
10^2
10^1
0.0
0.78
1.56
3.12
6.25
12.5
25
50
RsDPLA Concentration (ug/ml)
0 ng/ml MPL
100 ng/ml MPL
1,000 ng/ml MPL
10,000 ng/ml MPL

Figure 21. Comparative blocking of TNF secretion by RsMPLA. Results show the competetive blocking of LPS-stimulated TNF secretion from adherent peritoneal exudate macrophages by RsDPLA. The results are representative data derived from one of 16 separate experiments. Macrophages were pretreated with the indicated concentrations of RsDPLA for 2 hours prior to stimulation for 20 hours with the indicated concentrations of sLPS (Figure 21 A), ReLPS (Figure 21 B), DPLA (Figure 21 C), MPL (Figure 21 D).


A
TNF Activity (U/ml)
10^5
10^4
10^3
10^2
10^1
0.0
0.78
1.56
3.12
6.25
12.5
25
50
RsMPLA Concentration (ug/ml)
LPS 0 ng/ml
LPS 0.1 ng/ml
LPS 1.0 ng/ml
LPS 10 ng/ml


B
TNF Activity (U/ml)
10^5
10^4
10^3
10^2
10^1
0.0
0.78
1.56
3.12
6.25
12.5
25
50
RsMPLA Concentration (ug/ml)
0 ng/ml ReLPS
1.0 ng/ml ReLPS
10 ng/ml ReLPS
100 ng/ml ReLPS

C


TNF Activity (U/ml)
10^5
10^4
10^3
10^2
10^1
0.0
0.78
1.56
3.12
6.25
12.5
25
50
RsMPLA Concentration (ug/ml)
0 ng/ml DPLA
10 ng/ml DPLA
100 ng/ml DPLA
1,000 ng/ml DPLA

D

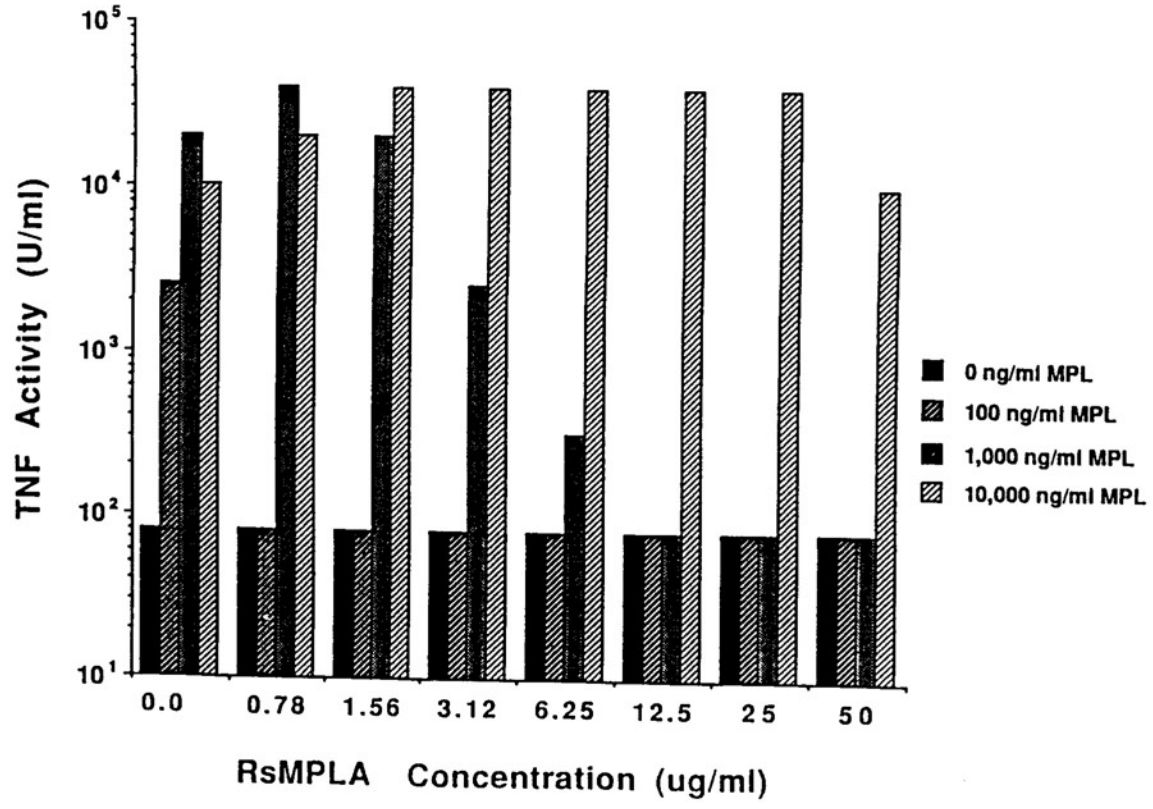

TNF Activity (U/ml)
10^5
10^4
10^3
10^2
10^1
0.0
0.78
1.56
3.12
6.25
12.5
25
50
RsMPLA Concentration (ug/ml)
0 ng/ml MPL
100 ng/ml MPL
1,000 ng/ml MPL
10,000 ng/ml MPL

Figure 22. Induction of *in vitro* tolerance by RsDPLA *versus* sLPS and MPL. Adherent peritoneal exudate macrophages were incubated for 20 hours with media alone, or the indicated concentrations of LPS derivatives. At the end of 20 hours incubation, cells were washed with fresh media, incubated for one hour, and "challenged" with 10 ng/ml *E coli* K235 LPS. Supernatants were collected after a second 20 hour incubation. Each data point represents the geometric mean $\pm$ standard error of the mean of three to eight separate experiments.

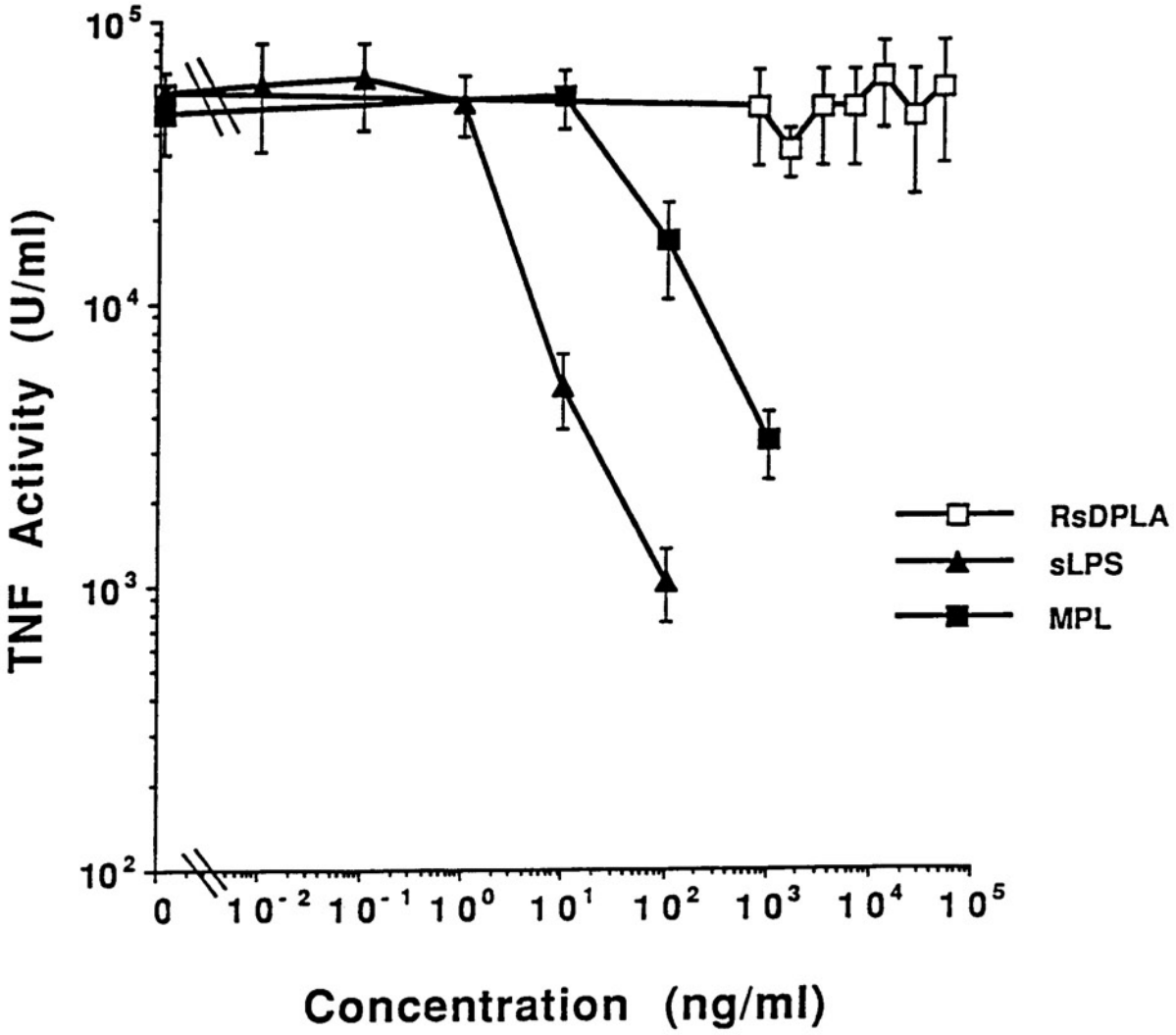
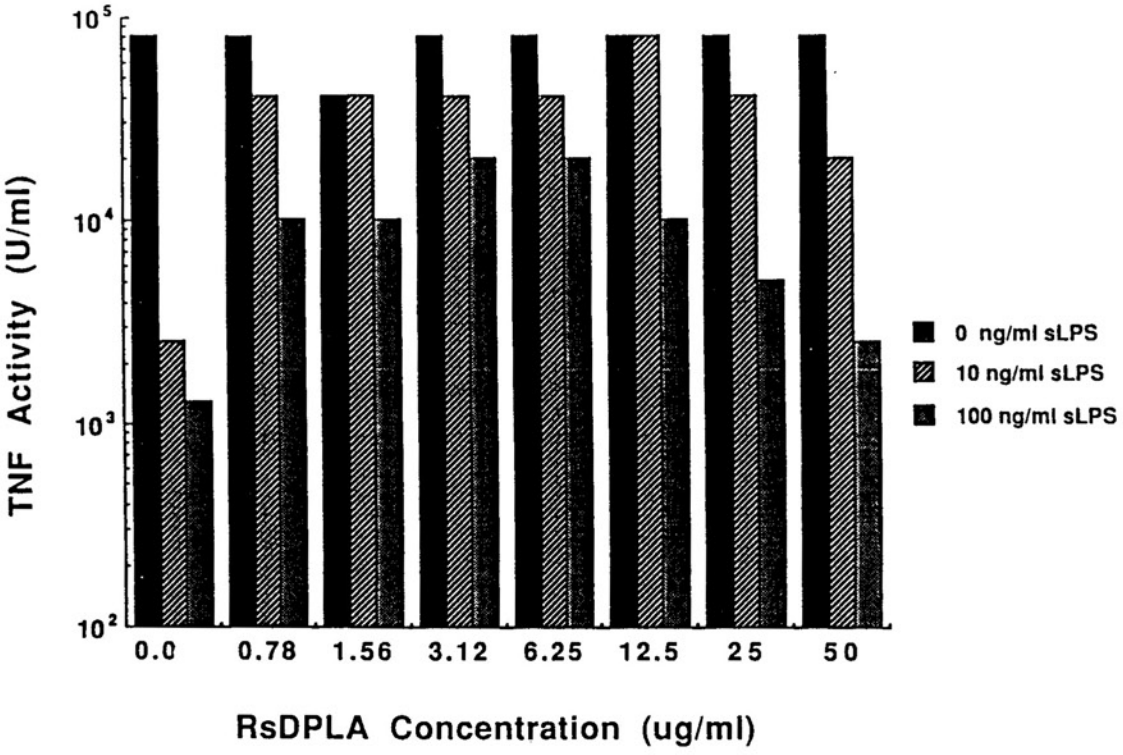
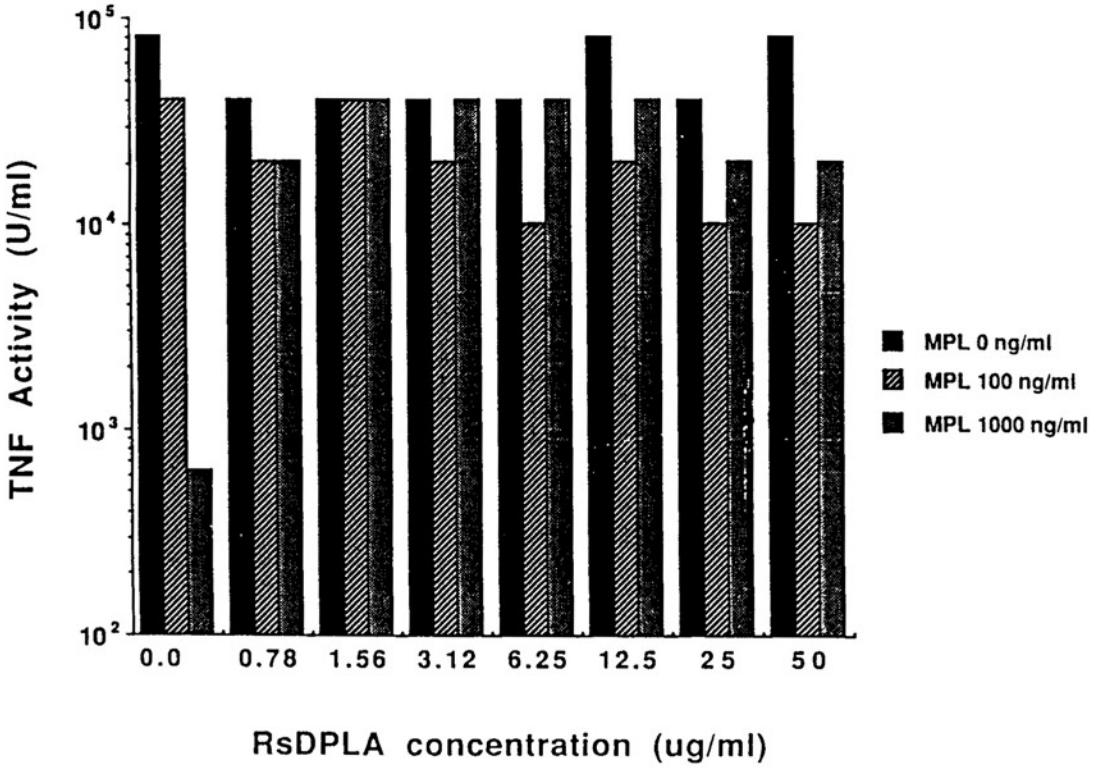

TNF Activity (U/ml)
10^5
10^4
10^3
10^2
0
10^-2
10^-1
10^0
10^1
10^2
10^3
10^4
10^5
Concentration (ng/ml)
RsDPLA
sLPS
MPL

Figure 23. *R. sphaeroides* DPLA reverses the induction of tolerance by sLPS and MPL. Adherent peritoneal exudate macrophages were pretreated with the indicated concentrations of RsDPLA for 2 hours, then incubated for 20 hours at with media alone, or the indicated dilutions of sLPS (Figure 23 A), or MPL (Figure 23 B). At the end of 20 hours incubation, cells were washed with fresh media, cultured for one hour, and "challenged" with 10 ng/ml sLPS . Supernatants were collected after a second 20 hours of incubation. The results are representive data derived from one of 16 separate experiments.

A


TNF Activity (U/ml)
10^5
10^4
10^3
10^2
0.0
0.78
1.56
3.12
6.25
12.5
25
50
RsDPLA Concentration (ug/ml)
0 ng/ml sLPS
10 ng/ml sLPS
100 ng/ml sLPS

B


TNF Activity (U/ml)
10^5
10^4
10^3
10^2
0.0
0.78
1.56
3.12
6.25
12.5
25
50
RsDPLA concentration (ug/ml)
MPL 0 ng/ml
MPL 100 ng/ml
MPL 1000 ng/ml

ng/ml MPL (Figure 23 B) resulted in dose-dependent tolerance to subsequent sLPS "challenge." When macrophages were pre-treated for 2 hours with increasing concentrations of RsDPLA (up to 6.25 μg/ml), tolerance induced by 10 ng/ml sLPS was completely reversed, and that induced by 100 ng/ml sLPS was partially reversed (Figure 23 A). Higher (> 25 μg/ml) concentrations of RsDPLA failed to over-ride tolerance induced by 100 ng/ml sLPS. Figure 23 B illustrates that RsDPLA was a more effective inhibitor of MPL-induced tolerance than sLPS-induced tolerance. As little as 0.78 μg/ml RsDPLA completely reversed the tolerance induced by 1,000 ng/ml MPL.

Comparison of AOAH induction by LPS and MPL. Acyloxyacyl hydrolase (AOAH) is cell-associated and its secretion into the circulation has not been documented. As an intracellular protein that binds and detoxifies LPS *in vivo*, AOAH has been demonstrated to recognize and remove acyloxyacyl groups from lipid A structures, converting them into compounds with structures similar to the lipid IV_A lipid A-precursor (Munford, 1986), with a resultant decrease in activity for human monocytes (Erwin *et al.*, 1991). AOAH is rather unique among enzymes, since it is able to distinguish and remove not only 2 and 2' N-linked, but also 3'-O-linked, acyloxyacyl groups (Erwin and Munford, 1990). Since AOAH has the ability to detoxify LPS within the cell, it was hypothesized that the basis of tolerance to LPS might rest on the ability of the cell to increase the amount or activity of AOAH after an initial exposure to LPS. AOAH might contribute to the lowered toxicity of MPL or its decreased efficiency as a "tolerogen" by deacylating it more efficiently. Alternately, MPL might induce more AOAH than LPS, which could result in more efficient detoxification of MPL by the cell. In the following experiments, AOAH activity induced by LPS *versus* MPL was monitored *in vitro*. First, peritoneal exudate macrophages were

treated either with medium or varying concentrations of LPS or MPL. Twenty hours later, cells were lysed and the cytoplasmic fractions assayed for enzyme activity. After twenty hours, there was a clear dose-dependent induction of AOAH activity by LPS and MPL, although MPL induced slightly less AOAH activity than LPS (Figure 24).

Second, the rate at which AOAH deacylated ^{3}H-LPS was determined for (1) untreated peritoneal exudate macrophages, (2) cells exposed to 100 ng/ml *E. coli* LPS (a concentration sufficient to induce *in vitro* tolerance) for 20 hours, then washed and reincubated with fresh media, (3) cells incubated in media for 20 hours which were then "challenged" with 10 ng/ml *E. coli* LPS for 20 hours, or (4) macrophages that were incubated with 100 ng/ml *E. coli* LPS for 20 hours and then were washed and "challenged" with 10 ng/ml *E.coli* LPS. All treatment groups were treated for 6 hours with ^{3}H-LPS, and then were washed with fresh medium to remove all non-adherent ^{3}H-LPS. Macrophage cultures were then reincubated. Cell lysates were harvested 4 hours later and the levels of ^{3}H fatty acids separated from the ^{3}H-LPS was quantified by ethanol-BSA precipitation of the ^{3}H-LPS remaining in the lysates. Free fatty acids were assayed in the ethanol supernatants and the rate of deacylation for each treatment group was calculated using the specific activity of the ^{3}H-LPS. Macrophages that had been treated with LPS once exhibited a higher rate of deacylation than untreated cells (Figure 25). The rate of deacylation for these cells stayed elevated even though the LPS was subsequently removed. The "challenge" dose alone (10 ng/ml LPS) induced approximately the same rate of deacylation as treatment with the 100 ng/ml (tolerizing) concentration. Macrophages that were treated with 100 ng/ml *E. coli* LPS and then challenged with 10 ng/ml LPS exhibited a rate of deacylation approximately 2-3-fold higher than untreated cells, and at a rate that was twice as great as would be expected from the challenge treatment alone.

Figure 24. The induction of acyloxyacyl hydrolase (AOAH) activity by LPS and MPL in peritoneal exudate macrophages. Peritoneal exudate macrophages were treated with the indicated concentrations of LPS and MPL for 20 hours, as indicated in the Materials and Methods. At the end of 20 hours incubation cells were lysed and lysates assayed for AOAH assay. Results are shown as ^{3}H-fatty acids released by AOAH activity in dpm and are the results of a representative experiment. The AOAH activity in cell lysates was relatively labile to freezing and thawing and depended upon protease inhibitors to preserve activity. Also, AOAH activity in lysates did not reflect the actual processing of LPS by the macrophages. In order to avoid these problems, ^{3}H-LPS was used in subsequent experiments to determine the rate of deacylation by AOAH.

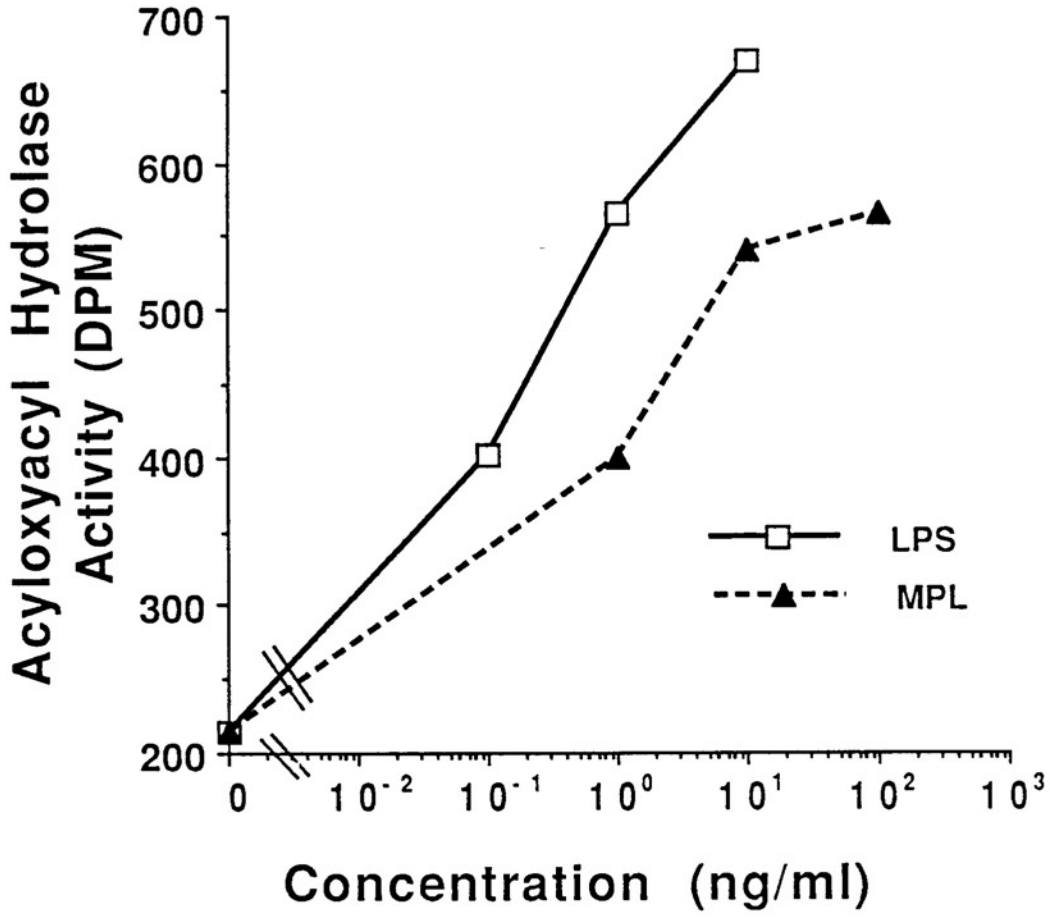
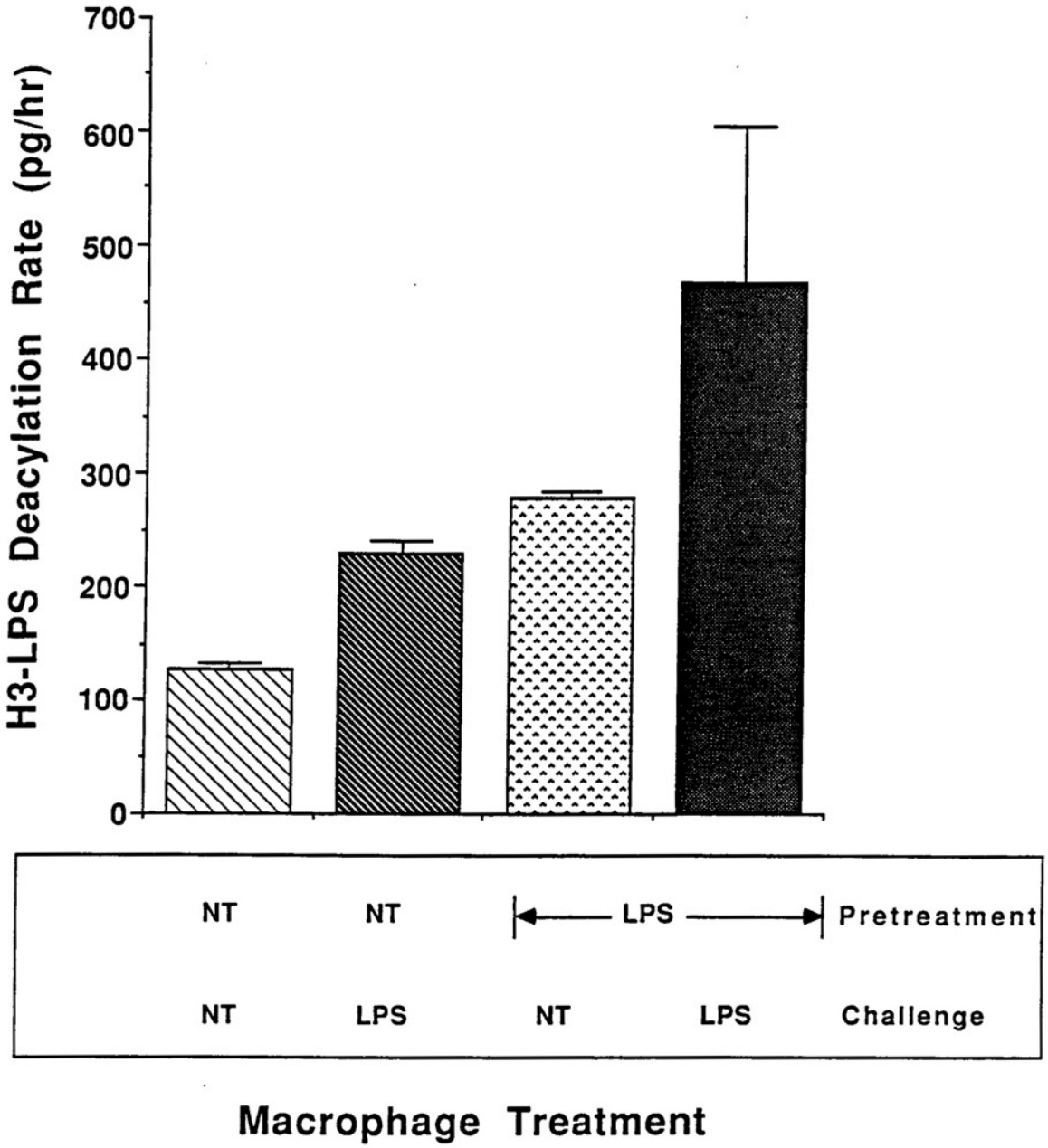

Acyloxyacyl Hydrolase Activity (DPM)
700
600
500
400
300
200
0
10-2
10-1
10 0
10 1
10 2
10 3
LPS
MPL
Concentration (ng/ml)

Figure 25. Comparison of AOAH activity in control and tolerized peritoneal exudate macrophages. Macrophage cultures were treated for 20 hours with either medium or 100 ng/ml *E. coli* LPS. After 20 hours incubation, cells were washed twice with fresh medium and then restimulated either with fresh medium or 10 ng/ml *E. coli* LPS, as indicated in the Materials and Methods. After a second 20 hour incubation, cells were pulsed with ^{3}H-LPS for 6 hours, then were washed to remove non-adherent ^{3}H-LPS. After another 4 hours of incubation (e.g., a total of 10 hours to deacylate the LPS), cells were lysed and subjected to extraction of the ^{3}H-fatty acids. Results are shown as the rate of deacylation per hour in pg/hr, and represent the results of one of two separate experiments.


H3-LPS Deacylation Rate (pg/hr)
700
600
500
400
300
200
100
0
NT
NT
LPS
Pretreatment
NT
LPS
NT
LPS
Challenge
Macrophage Treatment

LPS-INDUCIBLE GENE EXPRESSION

All of the studies described in the first two sections of this dissertation focused on *in vivo* responses to LPS *versus* MPL. Those studies were extended in the previous section to include an examination of the relative capacities of LPS and its derivatives to interact with macrophages *in vitro* to induce cytokine production in macrophages or to induce "tolerance" to further stimulation by LPS *in vitro*. Analysis of these same events at the molecular level was next sought to delineate further the structure to function relationships which are extant in macrophage-LPS interactions. Since the process of cytokine secretion is subject to a variety of levels of intracellular control, examination of steady-state mRNA was an additional means to view LPS-macrophage interactions from a vantage point intermediate in the biochemical pathway which is initiated by receptor engagement and finalized by cytokine production.

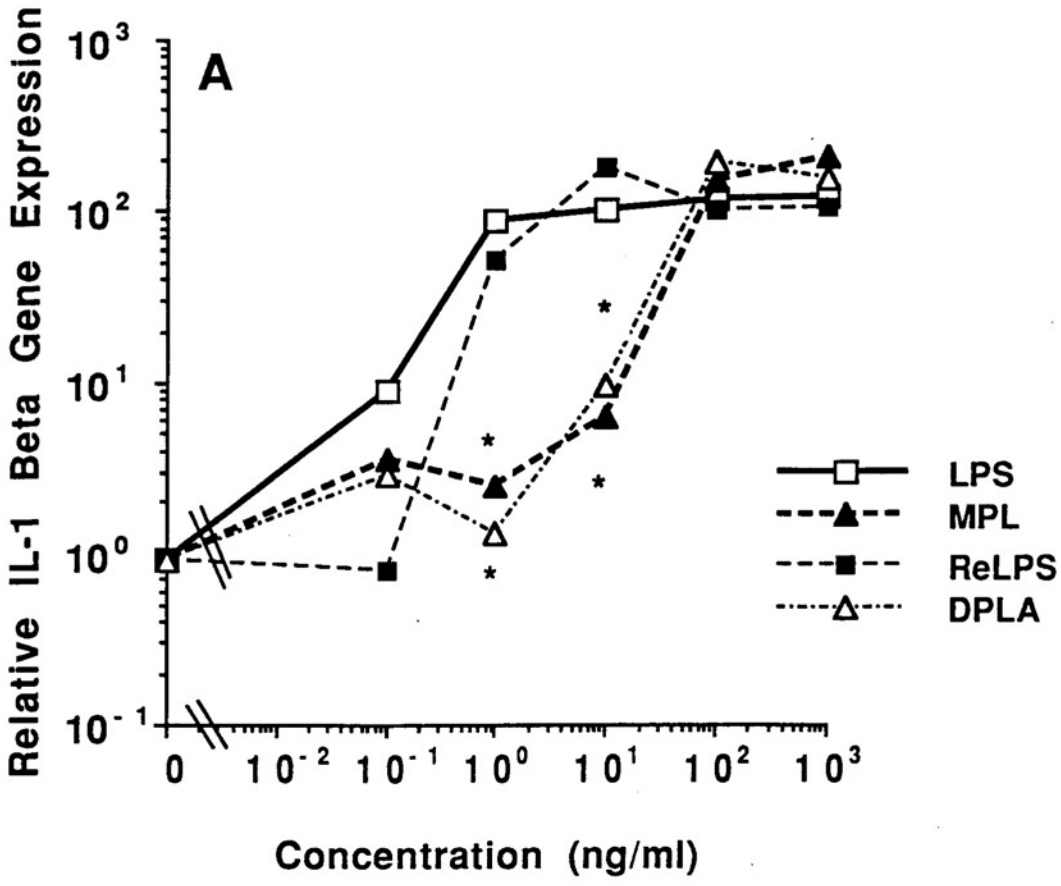
Recently, Tannenbaum *et al.* (1988) have cloned six LPS-inducible genes (designated D2, D3, D5, D7, D8 and C7) by differential screening of a cDNA library created from LPS-treated C57BL/6J mouse peritoneal exudate macrophages. These genes were all strongly induced by 3 hours of LPS stimulation and were classified as "early" genes, based on their kinetics and lack of inbition by cycloheximide. Gene C7 has subsequently been identified as the murine homologue of IP-10, first identified as an interferon-γ-inducible protein (Ohmori and Hamilton, 1990) and a member of the Platelet Factor 4 intercrine family of small chemotactic and proinflammatory molecules (Luster *et al.*, 1985). Gene D5 was identified as IL-1β (Ohmori *et al.*, 1990) and D3 appears to be a

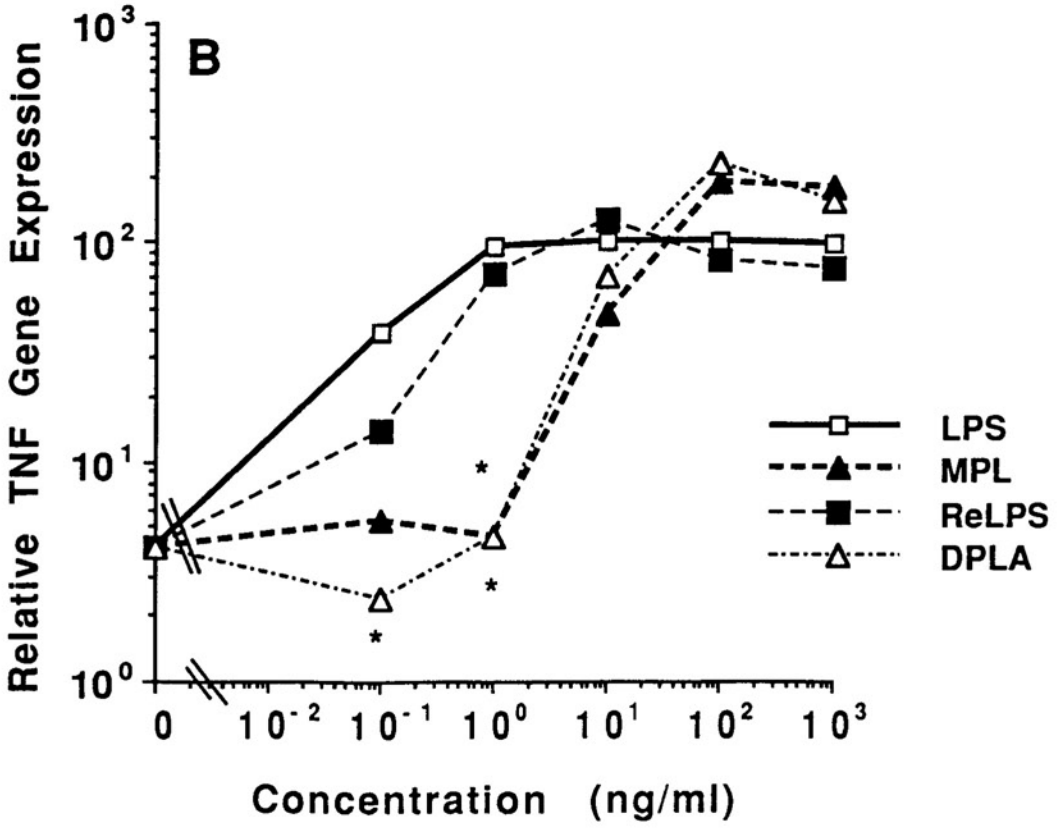
member of the interferon-α-inducible family of genes referred to as 202-204 (Choubey *et al.*, 1989). The remaining genes have yet to be identified. The availability of these probes presented a unique opportunity to investigate responses to endotoxin *in vitro*, at the genetic level. In this study, we have utilized this panel of six LPS-inducible genes, plus the gene for TNFα, to explore *in vitro* the inductive and tolerance-inducing effects of LPS and its toxic and non-toxic derivatives.

Dose response induction of LPS-inducible genes. The macrophage genes for TNFα and those first described by Tannenbaum *et al.* as LPS-inducible "early" genes were tentatively separated into groups based on similarities of their dose responses to smooth LPS, ReLPS, DPLA, and MPL. For both IL-1β (Figure 26 A) and TNFα (Figure 26 B), maximal LPS-induced steady-state mRNA (expressed by C57BL/6J macrophages after 4 hours of LPS stimulation) occurred at 1 ng/ml for smooth LPS and ReLPS; however, maximal DPLA- and MPL-induced gene expression required a 100-fold higher concentration. In spite of the similarities between LPS and ReLPS, and between DPLA and MPL, for the induction of TNFα steady-state mRNA, secreted TNF activity showed a somewhat different pattern of dose dependence: ReLPS, DPLA and MPL were 10-fold, 100-fold, and 1000-fold less potent than smooth LPS, respectively (Figure 26 C).

Genes for IP-10, D8, and D3 also exhibited a marked degree of similarity in an LPS dose response experiments (Figure 27 A-D). Maximal LPS-induced mRNA expression also occurred at 1 ng/ml for these genes, whereas DPLA- and MPL-induced mRNA expression was maximal at a 100-fold higher concentration. The concentration of ReLPS required to induce IP-10, D3, and D8 expression was intermediate between LPS-induced and DPLA/MPL-induced expression for these genes, but was not significantly different from LPS.

Figure 26. Dose response comparison of IL-1β (Figure 26 A) and TNFα (Figure 26 B) steady-state mRNA and secreted TNF activity (Figure 26 C) after 4 hours of stimulation by LPS, ReLPS, DPLA and MPL. Results for IL-1β and TNFα mRNA expression (Figures 26 A and 26 B) represent compiled PhosphorImager and scanning densitometer data measurements of total RNA on Northern blots from 2-4 separate experiments per treatment concentration. Relative gene expression has been normalized to the expression of β-actin by dividing the volume measurement for each gene by the volume measurement for β-actin in the same lane (gene/β–actin ratio). Measurements were then normalized to the maximal expression induced by 10 ng/ml *E. coli* K235 LPS (100%) after 4 hours of stimulation, by dividing the gene/β-actin ratio for each treatment by the gene/β-actin ratio for the 10 ng/ml LPS challenge, as described in the Materials and Methods. Asterisks indicate expression values significantly different ($p < 0.005$) from LPS-induced expression after 4 hours after stimulation, as determined by statistical analysis of Least Squares Means comparison of the compiled log transformed data. The graphed values are the computer generated Least Squares Means. Data were analysed by Mr. Jian-Zheng Zhou of the University of Maryland Department of Animal Science and Dr. Lawrence Douglass, statistician of the University of Maryland Department of Statistics using IBM format SAS software. Figure 26 C. Comparison of secreted TNF activity after 4 hours of stimulation by LPS, MPL, ReLPS, and DPLA. Results represent the arithmetic mean of 4 - 8 separate experiments. Error bars are the standard error of the mean. Asterisks indicate TNF values significantly different from LPS-induced TNF after 4 hours of stimulation, as determined by Student's *t* test ($p< 0.05$).


A
Relative IL-1 Beta Gene Expression
10^3
10^2
10^1
10^0
10^{-1}
0
10^{-2}
10^{-1}
10^0
10^1
10^2
10^3
Concentration (ng/ml)
LPS
MPL
ReLPS
DPLA


B
Relative TNF Gene Expression
10^3
10^2
10^1
10^0
0
10^{-2}
10^{-1}
10^0
10^1
10^2
10^3
Concentration (ng/ml)
LPS
MPL
ReLPS
DPLA

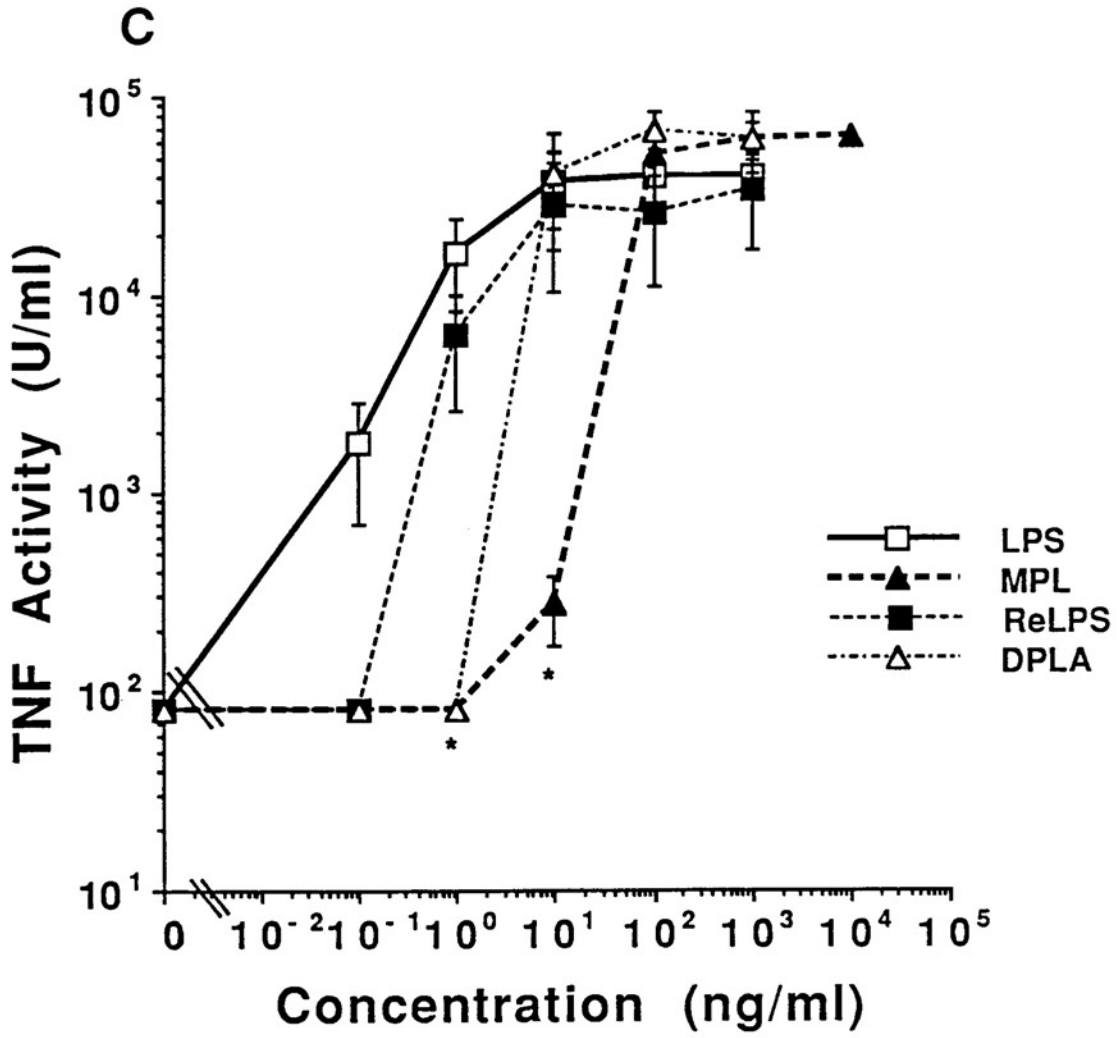
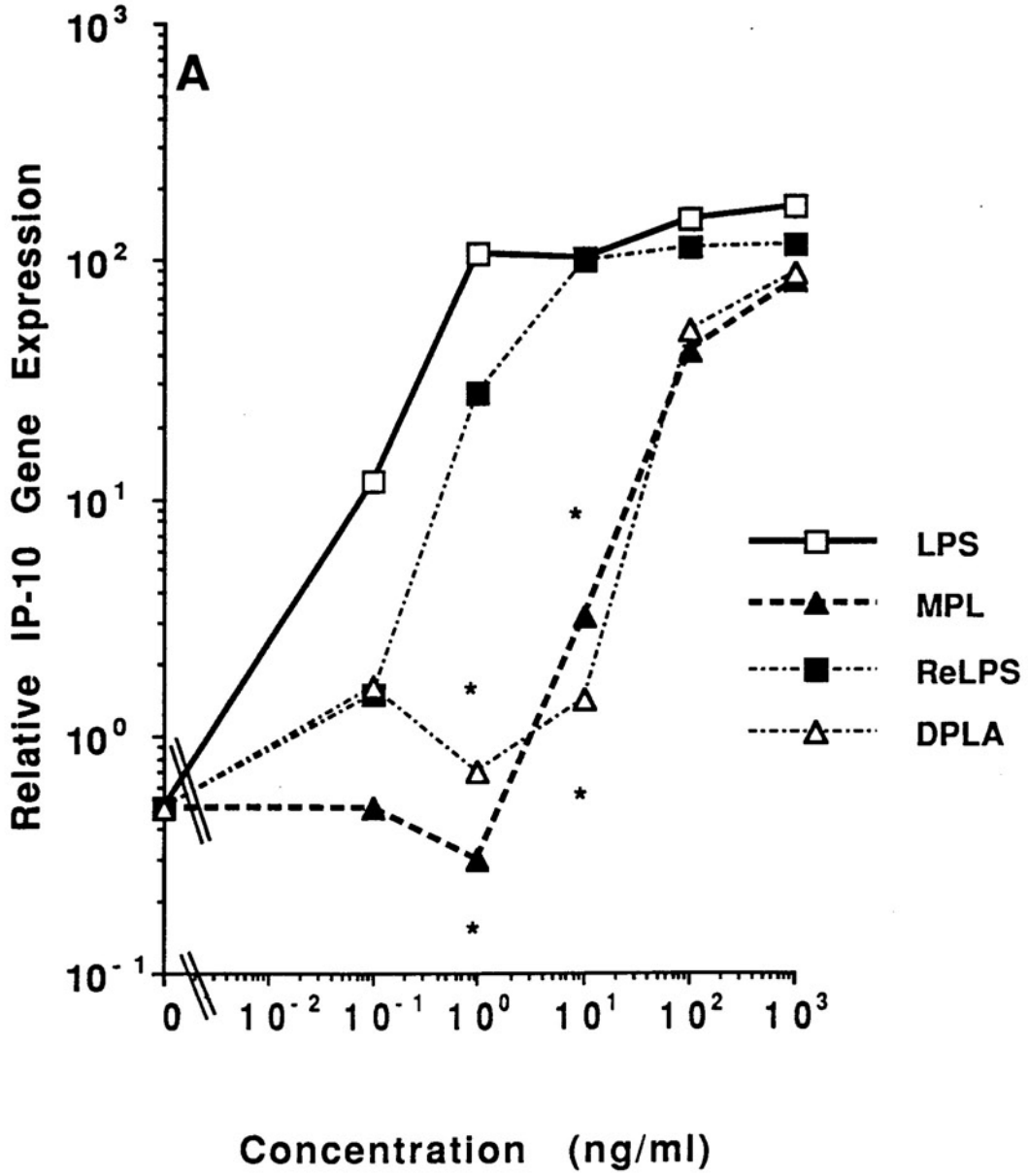
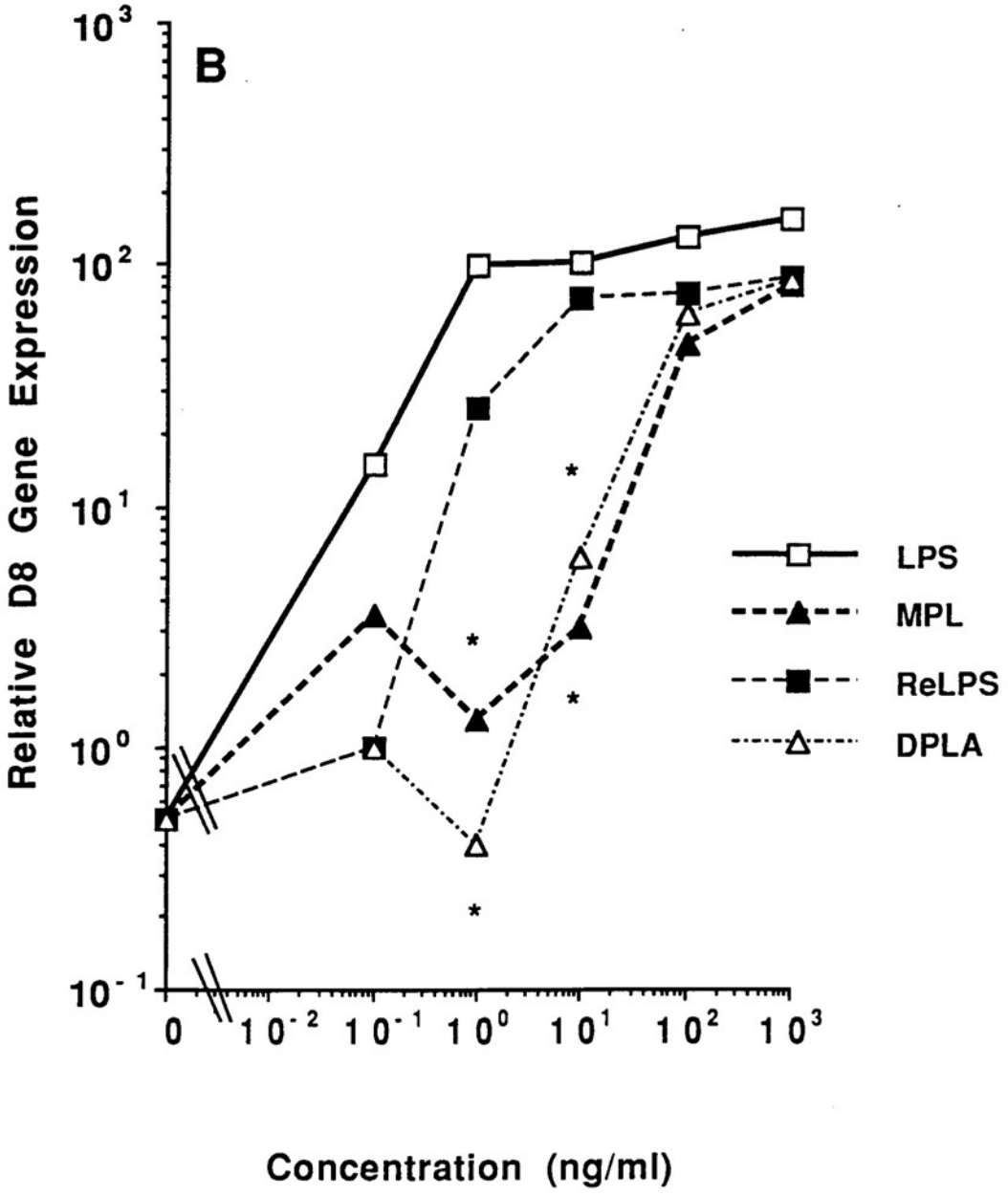
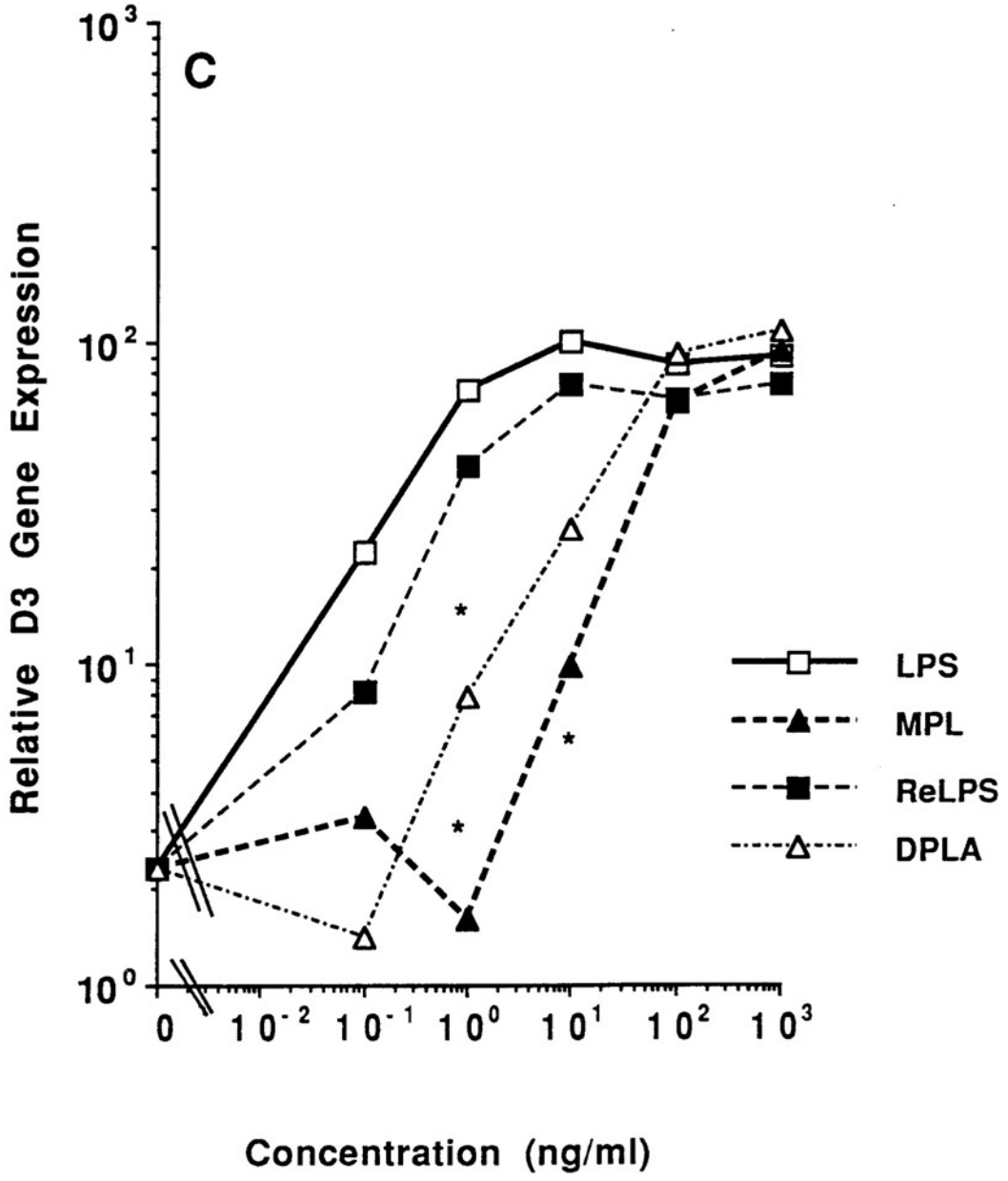

C
TNF Activity (U/ml)
10^5
10^4
10^3
10^2
10^1
0
10^{-2} 10^{-1} 10^0 10^1 10^2 10^3 10^4 10^5
Concentration (ng/ml)
LPS
MPL
ReLPS
DPLA
*
*

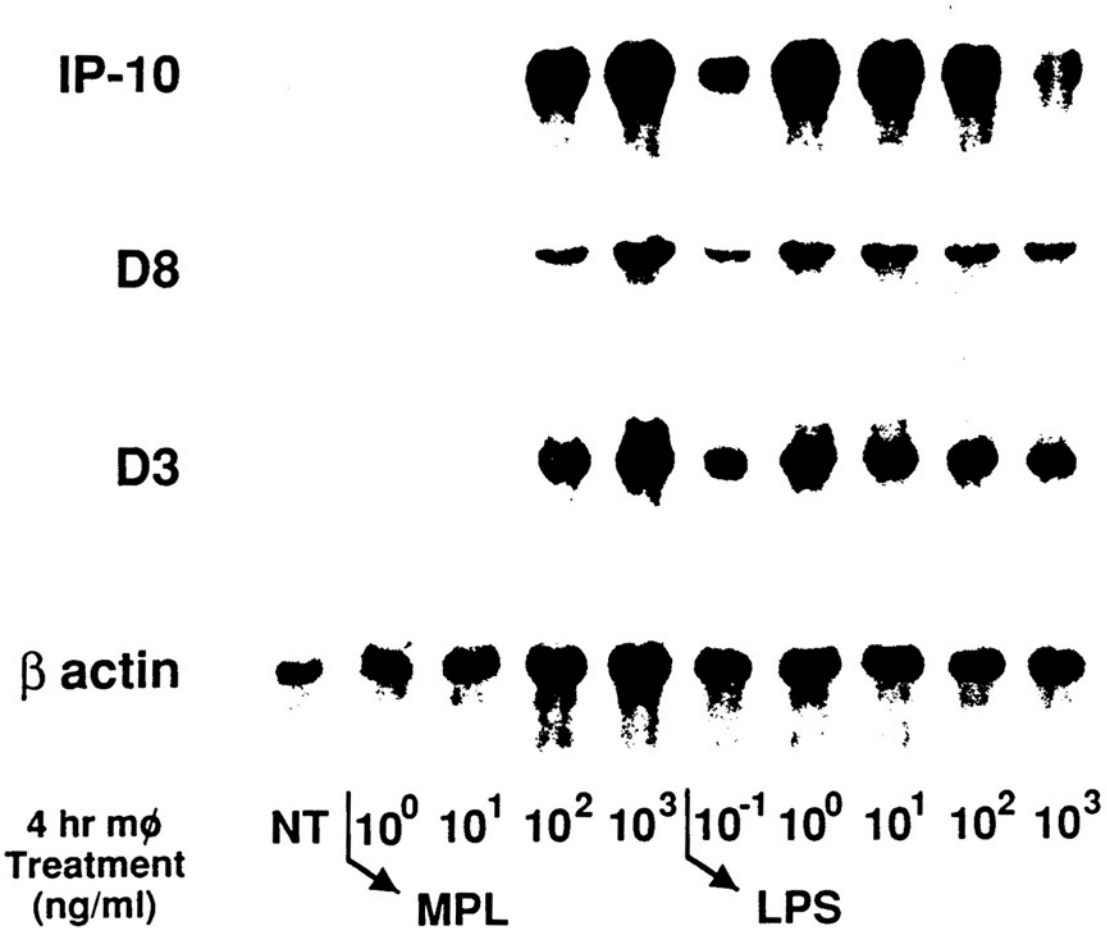
Figure 27. Dose response comparison of IP-10 (Figure 27 A), D8 (Figure 27 B) and D3 (Figure 27 C) steady-state mRNA after 4 hours of stimulation by LPS, ReLPS, DPLA and MPL. Results represent PhosphorImager and scanning densitometer data measurements of total RNA on Northern blots from 2-4 separate experiments per treatment concentration. Relative gene expression has been normalized to the expression of β-actin by dividing the volume measurement for each gene by the volume measurement for β-actin in the same lane (gene/β-actin ratio). Measurements were then normalized to the maximal expression induced by 10 ng/ml *E. coli* K235 LPS (100%) after 4 hours of stimulation, by dividing the gene/β-actin ratio for each treatment by the gene/β-actin ratio for the 10 ng/ml challenge, as described in the Materials and Methods. Asterisks indicate expression values significantly different ($p < 0.005$) from LPS-induced expression at 4 hours after stimulation, as determined by statistical analysis of Least Squared Means comparison of the compiled log transformed data. The graphed values are the computer generated Least Squares Means. Data were analysed by Mr. Jian-Zheng Zhou of the University of Maryland Department of Animal Science and Dr. Lawrence Douglass of the University of Maryland Department of Statistics using IBM format SAS software. Figure 27 D. Composite of autoradiographs of Northern blots from a single representative experiment depicting the comparative dose responses of IP-10, D8, and D3 for LPS *versus* MPL steady-state mRNA. The expression of the "housekeeping gene," β–actin, is included for comparative purposes. Concentrations of LPS and MPL listed below the figure are in ng/ml.


A
Relative IP-10 Gene Expression
10^3
10^2
10^1
10^0
10^{-1}
0
10^{-2}
10^{-1}
10^0
10^1
10^2
10^3
Concentration (ng/ml)
LPS
MPL
ReLPS
DPLA


B
Relative D8 Gene Expression
10^3
10^2
10^1
10^0
10^{-1}
0
10^{-2}
10^{-1}
10^0
10^1
10^2
10^3
Concentration (ng/ml)
LPS
MPL
ReLPS
DPLA
*
*
*
*


C
Relative D3 Gene Expression
10^3
10^2
10^1
10^0
0
10^{-2}
10^{-1}
10^0
10^1
10^2
10^3
Concentration (ng/ml)
LPS
MPL
ReLPS
DPLA

D

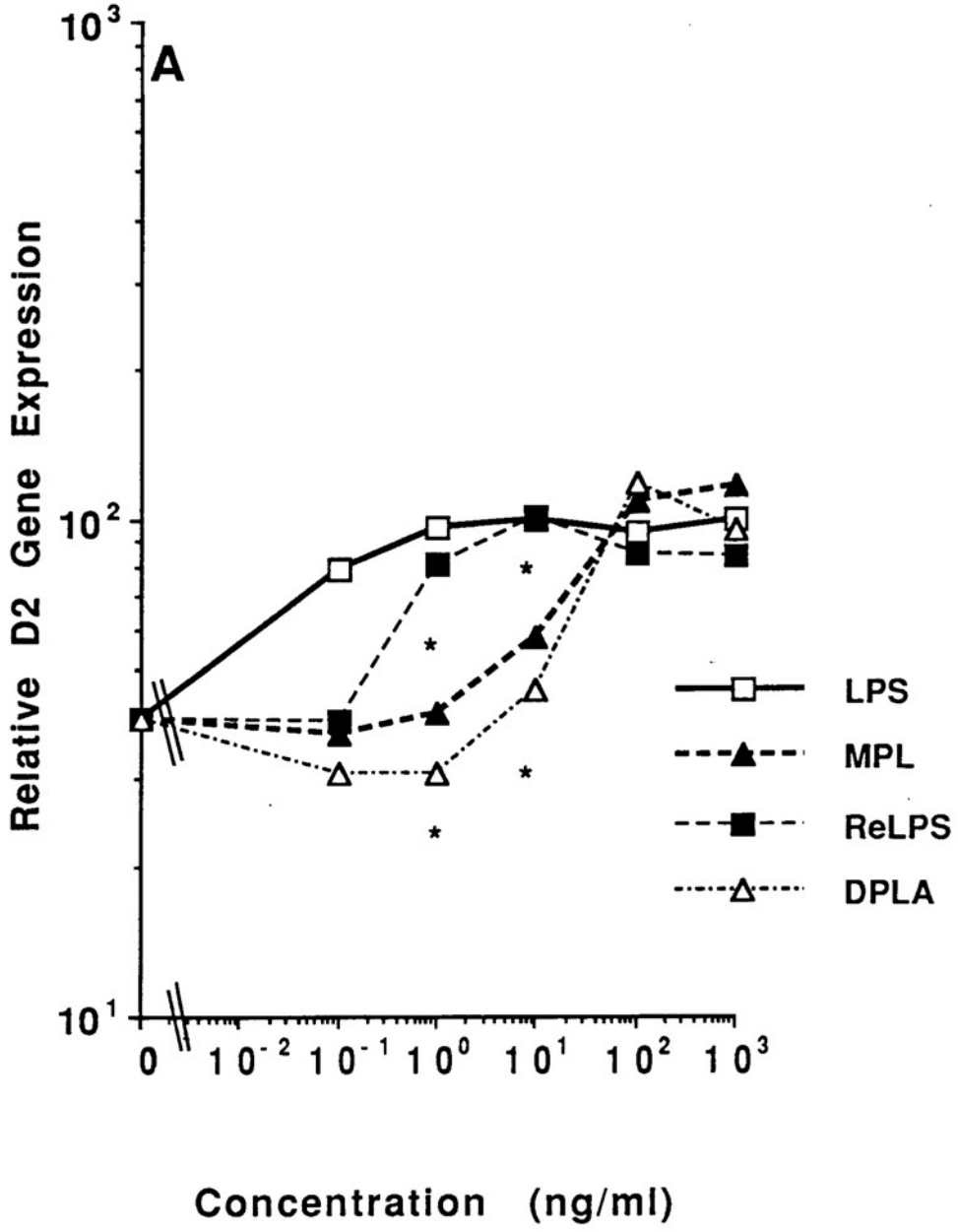

IP-10
D8
D3
β actin
4 hr mϕ Treatment (ng/ml)
NT
10^0
10^1
10^2
10^3
10^-1
10^0
10^1
10^2
10^3
MPL
LPS

The LPS-inducible genes designated D2 and D7 exhibited a third dose response pattern. Genes D2 and D7 showed relatively high constitutive expression in comparison to the other genes examined, which by PhosphorImager measurement represented approximately 40% of the expression induced by 10 ng/ml LPS after 4 hours of stimulation (Figures 28 A, B). MPL and DPLA proved to be 1,000-fold less potent than LPS in the induction of these two genes, and ReLPS was similar to though not significantly less potent than LPS, as it was for induction of IP-10, D8, and D3 steady-state mRNA.

Time course for the induction of LPS-inducible genes. The panel of LPS-inducible genes examined could also be subdivided into groups on the basis of the time course pattern stimulated by an excess (1,000 ng/ml) of LPS and each LPS derivative *in vitro*. For example, TNFα and IL-1β mRNA were induced maximally by LPS within 1-2 hour after stimulation (1 hour for TNFα and 2 hours for IL-1β) and were reduced to near background level within 12 hours (Figures 29 A, B). For these 2 genes, the peak expression induced by MPL was reproducibly later and more sustained than that induced by LPS, although the maximal levels of expression induced by MPL were not found to be different from that induced by LPS (Figure 30). The time courses for ReLPS- and DPLA-induced TNFα (Figure 29 A) and IL-1β (not shown) were indistinguishable from the LPS time course.

In contrast to the shift in kinetics for MPL-induced TNFα and IL-1β steady-state mRNA, no such shift was observed for MPL-induced IP-10, D8, and D3 (Figures 31 A-C). Maximal expression of IP-10 and D8 occurred 4 hours post-stimulation for all four structures examined, but IP-10 mRNA levels (Figure 31 A) declined with time, whereas D8 (Figure 31 B), once induced maximally, declines more slowly over the duration of the 12 hour time course. Although

Figure 28. Dose response comparison of D2 (Figure 28 A), and D7 (Figure 28 B) steady-state mRNA after 4 hours of stimulation by LPS, ReLPS, DPLA and MPL for 4 hours. Results represent PhosphorImager and scanning densitometer data measurements of total RNA on Northern blots from 3-4 separate experiments per treatment concentration. Relative gene expression has been normalized to the expression of β-actin by dividing the volume measurement for each gene by the volume measurement for β-actin in the same lane (gene/β–actin ratio). Measurements were then normalized to the maximal expression induced by 10 ng/ml *E. coli* K235 LPS (100%) after 4 hours of stimulation, by dividing the gene/β-actin ratio for each treatment by the gene/β-actin ratio for the 10 ng/ml challenge, as described in the Materials and Methods. Asterisks indicate expression values significantly different ($p < 0.005$) from LPS-induced expression at 4 hours after stimulation, as determined by statistical analysis of Least Squares Means comparison of the compiled log transformed data. The graphed values are the computer generated Least Squares Means. Data were analysed by Mr. Jian-Zheng Zhou of the University of Maryland Department of Animal Science and Dr. Lawrence Douglass of the University of Maryland Department of Statistics using IBM format SAS software.


A
Relative D2 Gene Expression
10^3
10^2
10^1
0
10^{-2}
10^{-1}
10^0
10^1
10^2
10^3
LPS
MPL
ReLPS
DPLA
Concentration (ng/ml)

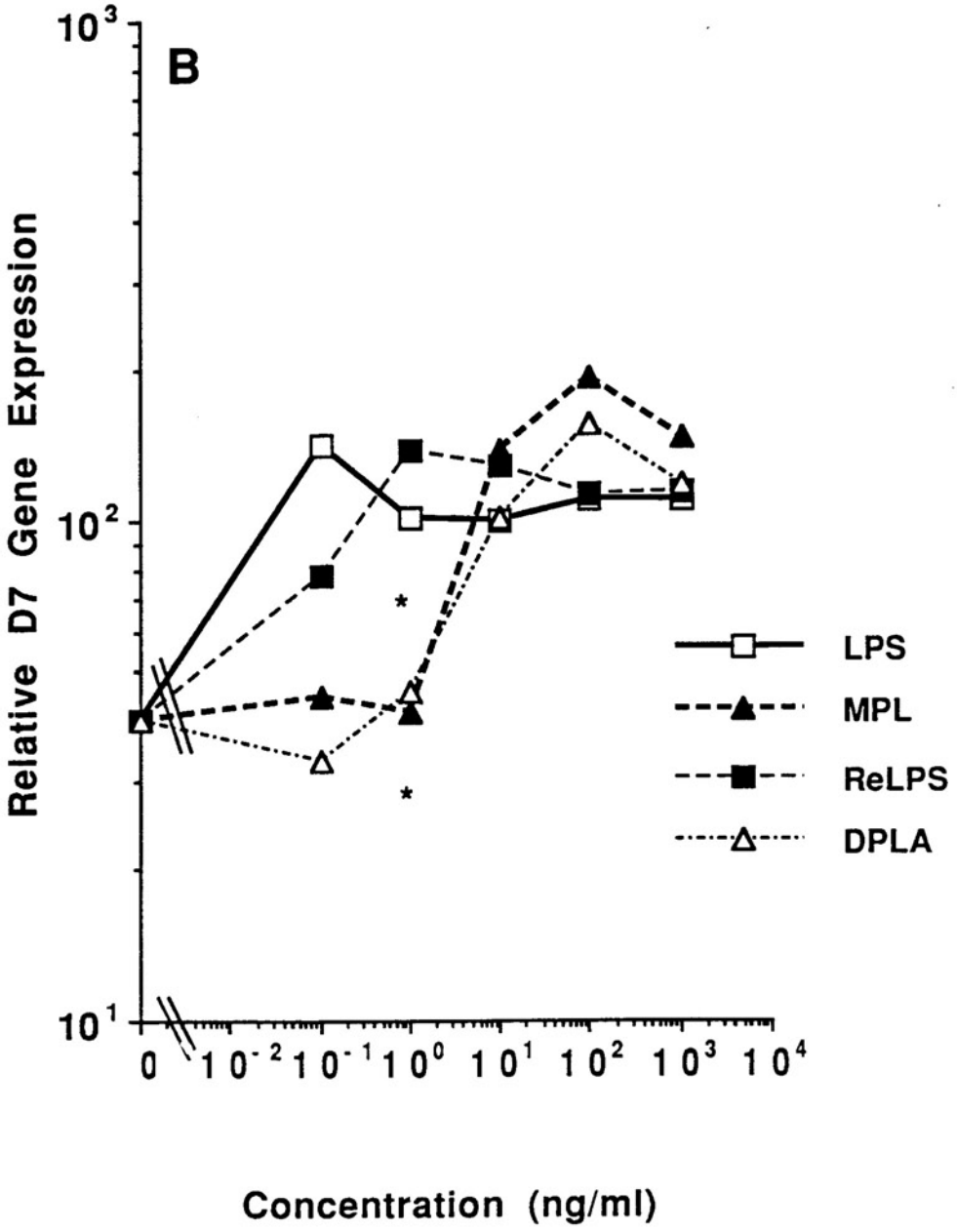

B
Relative D7 Gene Expression
10^3
10^2
10^1
0 10^{-2} 10^{-1} 10^0 10^1 10^2 10^3 10^4
Concentration (ng/ml)
LPS
MPL
ReLPS
DPLA
*
*

Figure 29. Time course of TNFα (Figure 29 A), and IL-1β (Figure 29 B) steady-state mRNA expression over 12 hours of stimulation by LPS, ReLPS, DPLA, and MPL. Results represent PhosphorImager and scanning densitometer data measurements of total RNA on Northern blots from 2 (ReLPS and DPLA), 3 (MPL), or 5 (LPS) separate time course experiments. Relative gene expression has been normalized to the expression of β-actin by dividing the volume measurement for each gene by the volume measurement for β-actin in the same lane (gene/β–actin ratio). Measurements were then normalized to the maximal expression induced by 1000 ng/ml *E. coli* K235 LPS (100%) stimulation, by dividing the gene/β-actin ratio for each treatment by the gene/β-actin ratio for the 1,000 ng/ml treatment, as described in the Materials and Methods. The graphed values are the computer generated Least Squares Means. Data were analysed by Mr. Jian-Zheng Zhou of the University of Maryland Department of Animal Science and Dr. Lawrence Douglass of the University of Maryland Department of Statistics using IBM format SAS software.

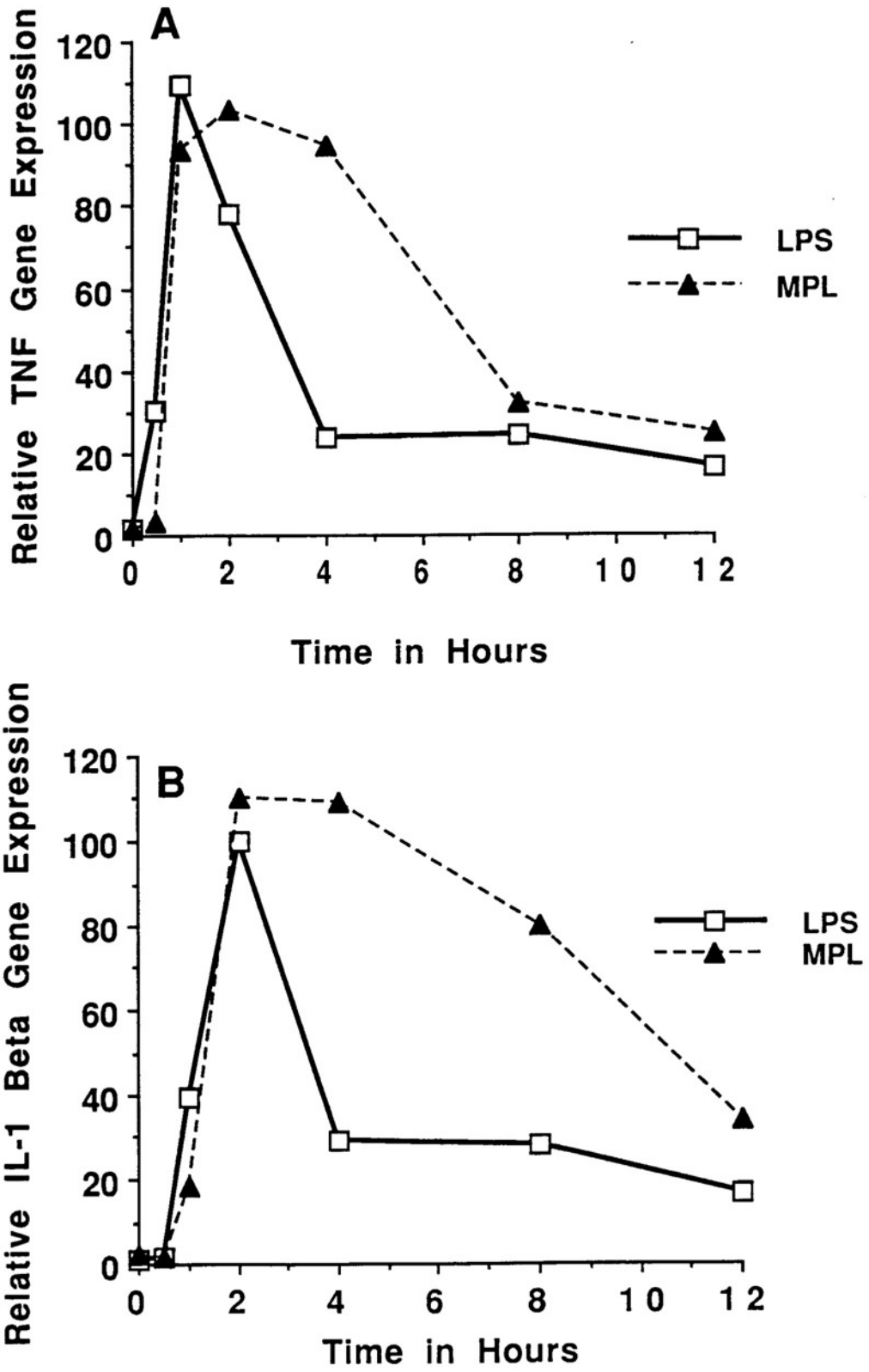
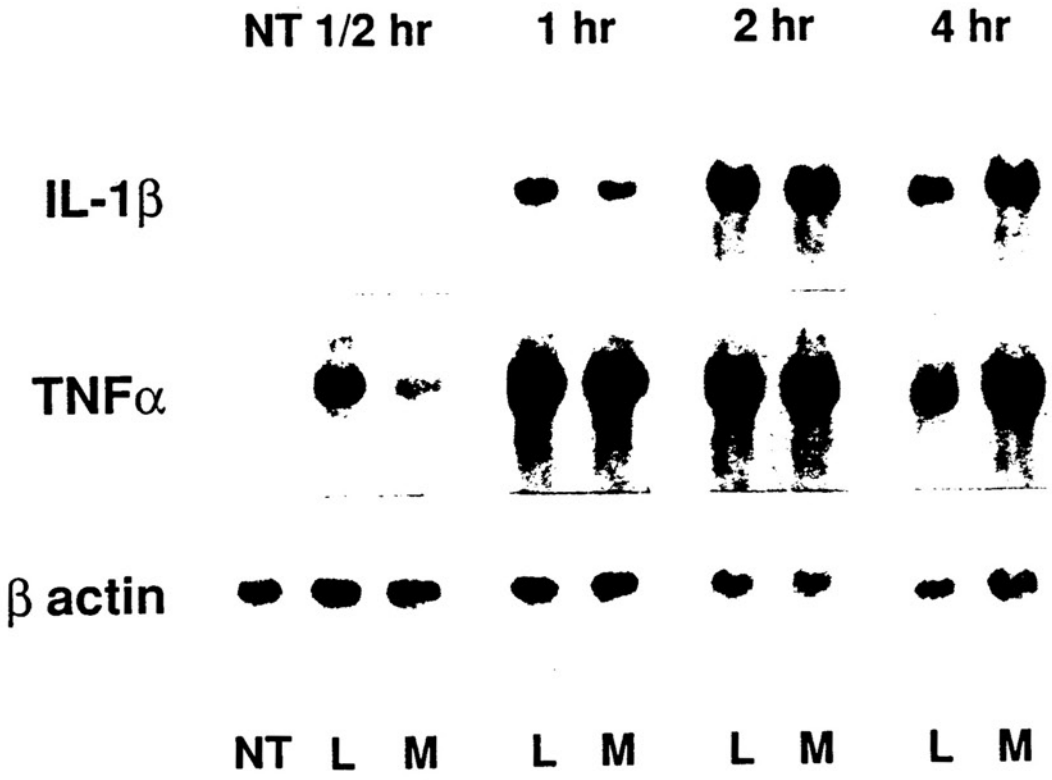
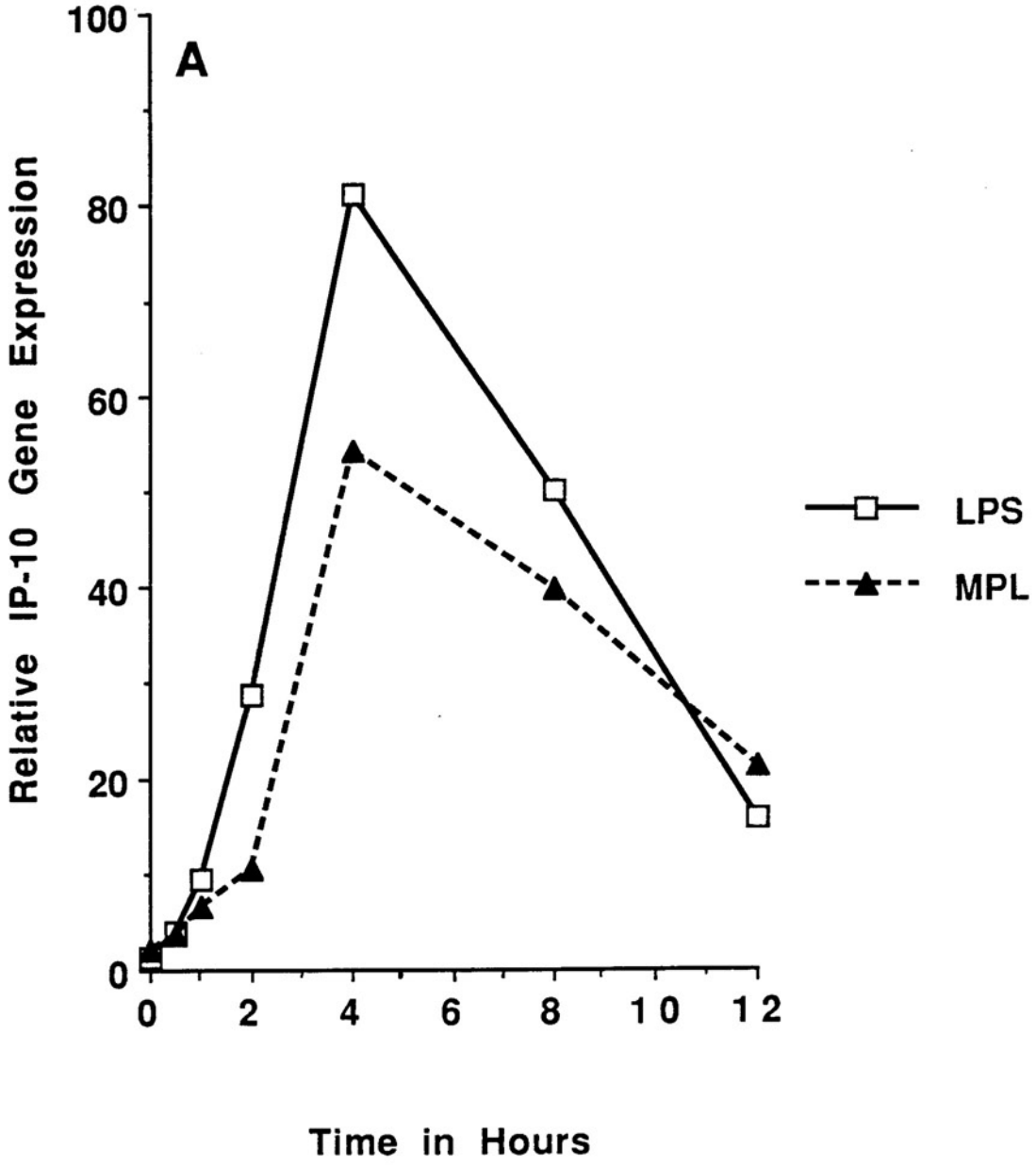

A
Relative TNF Gene Expression
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Time in Hours
LPS
MPL
B
Relative IL-1 Beta Gene Expression
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Time in Hours
LPS
MPL

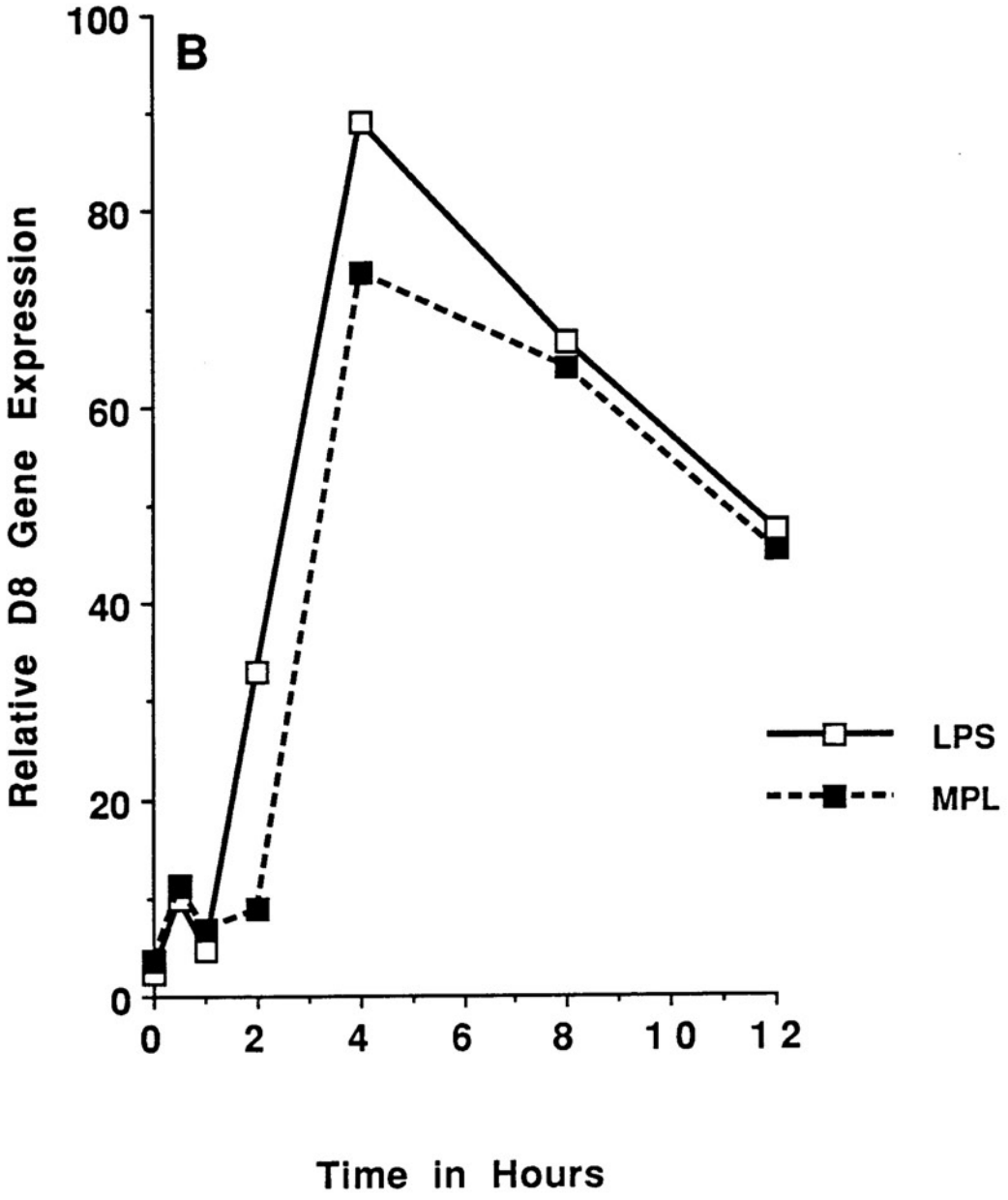
Figure 30. Composite autoradiograph depicting time course of LPS *versus* MPL for IL-1β (Figure 30 A) and TNF α (Figure 30 B). Composite autoradiographs of Northern blots show comparison of time course for IL-1β and TNFα steady-state mRNA for a single representative experiment in which peritoneal exudate macrophages were stimulated by 1,000 ng/ml LPS and MPL. The expression of the "housekeeping gene," β actin, is shown for comparison for the same lanes. "NT" indicates cells not treated; "L" designates LPS treatment; "M" indicates MPL treatment.

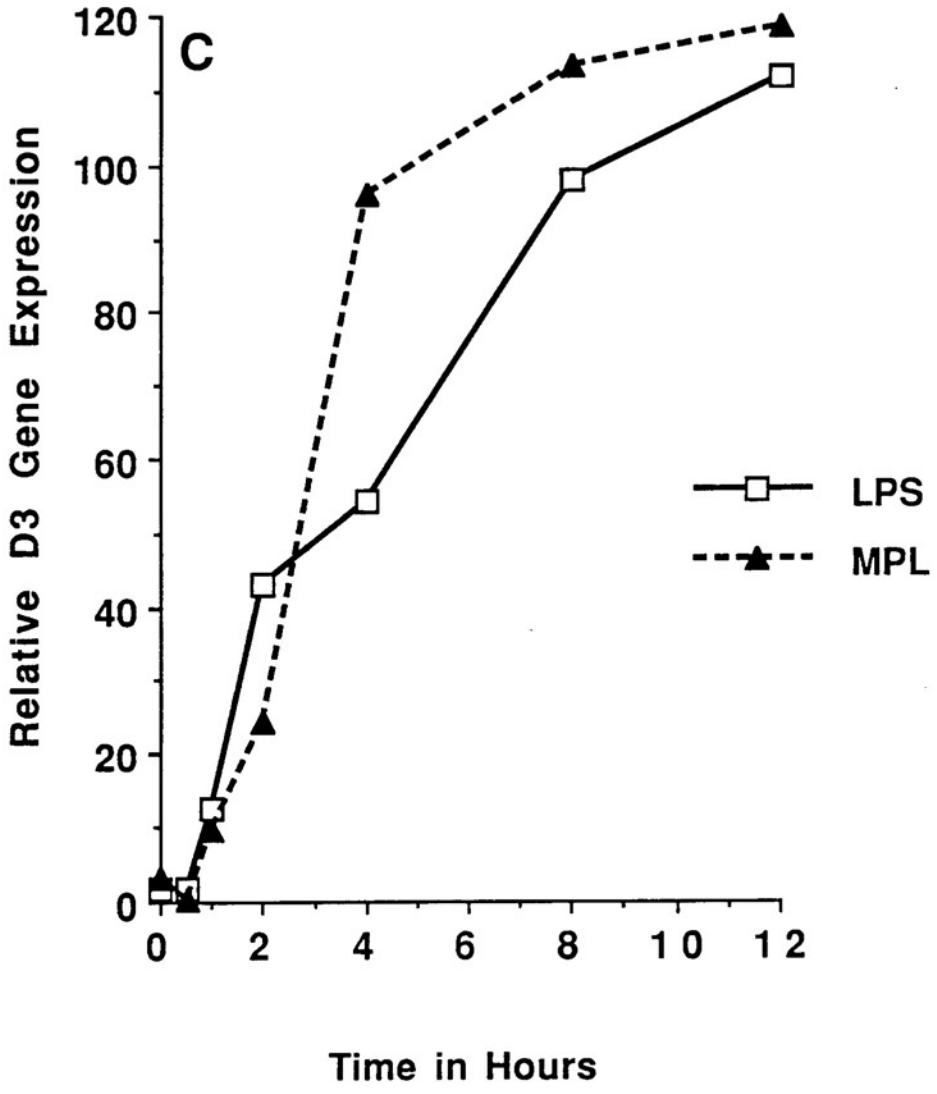


L = 1,000 ng/ml LPS
M = 1,000 ng/ml MPL

Figure 31. Time course of IP-10 (Figure 31 A), D8 (Figure 31 B), and D3 (Figure 31 C) steady-state mRNA expression over 12 hours of stimulation by LPS *versus* MPL. Results represent PhosphorImager and scanning densitometer data measurements of total RNA on Northern blots from 3-5 separate time course experiments. Relative gene expression has been normalized to the expression of β-actin by dividing the volume measurement for each gene by the volume measurement for β-actin in the same lane (gene/β–actin ratio). Measurements were then normalized to the maximal expression induced by 1,000 ng/ml *E. coli* K235 LPS (100%) stimulation, by dividing the gene/β-actin ratio for each treatment by the gene/β-actin ratio for the 1,000 ng/ml treatment, as described in the Materials and Methods. The graphed values are the computer generated Least Squares Means. Data were analysed by Mr. Jian-Zheng Zhou of the University of Maryland Department of Animal Science and Dr. Lawrence Douglass of the University of Maryland Department of Statistics using IBM format SAS software.


A
100
80
60
40
20
0
Relative IP-10 Gene Expression
0
2
4
6
8
10
12
Time in Hours
LPS
MPL


B
100
80
60
40
20
0
Relative D8 Gene Expression
0
2
4
6
8
10
12
Time in Hours
LPS
MPL

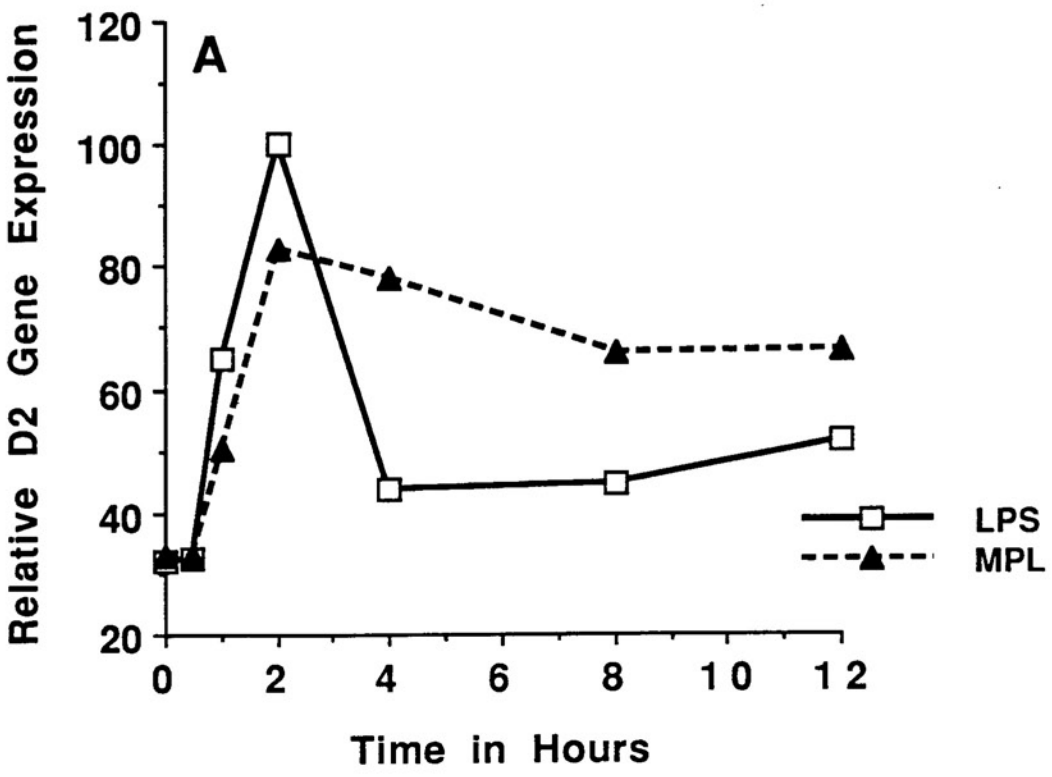

C
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Relative D3 Gene Expression
Time in Hours
LPS
MPL

the time course for D3 gene expression (Figure 31 C) was similar to that of IP-10 and D8, maximal D3 gene expression occurred somewhat later, but, remained elevated at 8 to 12 hours following stimulation. ReLPS and DPLA also stimulated D3 indistinguishably from MPL and LPS (data not shown).

The constitutively expressed genes, D2 and D7, were considerably more variable in their inducibility than the others, although they exhibited peak expression typically between 2 and 4 hours after LPS stimulation (Figure 32 A, B). Peak mRNA expression stimulated by LPS was not different in timing or magnitude from the D2 or D7 expression induced by the LPS derivatives, though MPL-induced D2 and D7 expression was sustained in a similar fashion to IL-1β and TNFα.

LPS-inducible gene expression in LPS- or MPL-pretreated macrophages stimulated by LPS "challenge". We have previously demonstrated that significantly more MPL than LPS is required *in vivo* and *in vitro* to induce a state of "tolerance" (Madonna and Vogel, 1985, 1986; Madonna *et al.*, 1986, Henricson *et al.*, 1990). To examine gene induction in pretreated cultures, adherent peritoneal exudate macrophages were first exposed to medium only or the indicated concentrations of LPS or MPL for 20 hours. Cultures were then washed and restimulated with 10 ng/ml LPS for 4 hours prior to RNA isolation. The 10 ng/ml "challenge" concentration of LPS was based on the maximal LPS gene expression data presented in Figures 26 - 28. LPS challenge of cultures pretreated with LPS or MPL resulted in TNFα, IL-1β, and D3 gene induction (Figure 33 A-C) which exhibited the following general features: at concentrations of LPS or MPL below that which renders cells LPS-hyporesponsive *in vitro* (e.g., < 1 ng/ml and < 100 ng/ml, respectively), a significant increase in gene induction was observed. In these three genes, expression was initially

Figure 32. Time course of D2 (Figure 32 A) and D7 (Figure 32 B) steady-state mRNA expression over 12 hours of stimulation by LPS *versus* MPL. Results represent PhosphorImager and scanning densitometer data measurements of total RNA on Northern blots from 3-5 separate time course experiments. Relative gene expression has been normalized to the expression of β-actin by dividing the volume measurement for each gene by the volume measurement for β-actin in the same lane (gene/β–actin ratio). Measurements were then normalized to the maximal expression induced by 1,000 ng/ml *E. coli* K235 LPS (100%) stimulation, by dividing the gene/β-actin ratio for each treatment by the gene/β-actin ratio for the 1,000 ng/ml treatment, as described in the Materials and Methods. The graphed values are the computer generated Least Squares Means. Data were analysed by Mr. Jian-Zheng Zhou of the University of Maryland Department of Animal Science and Dr. Lawrence Douglass of the University of Maryland Department of Statistics using IBM format SAS software.


A
Relative D2 Gene Expression
120
100
80
60
40
20
0
2
4
6
8
10
12
Time in Hours
LPS
MPL

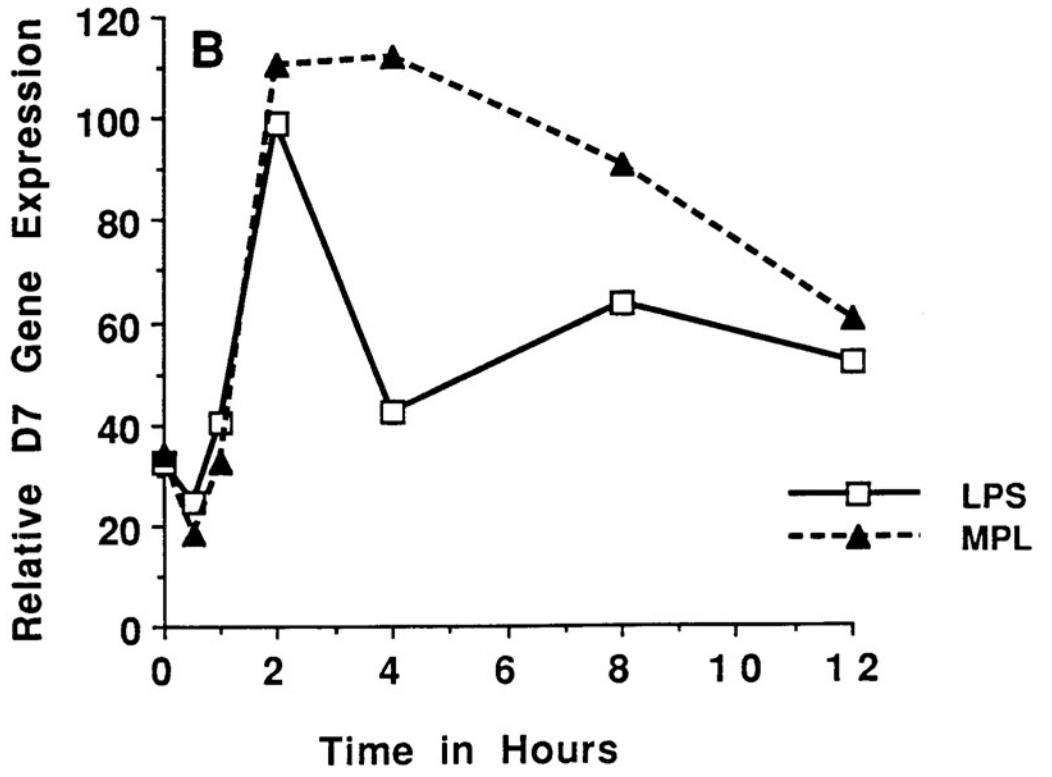
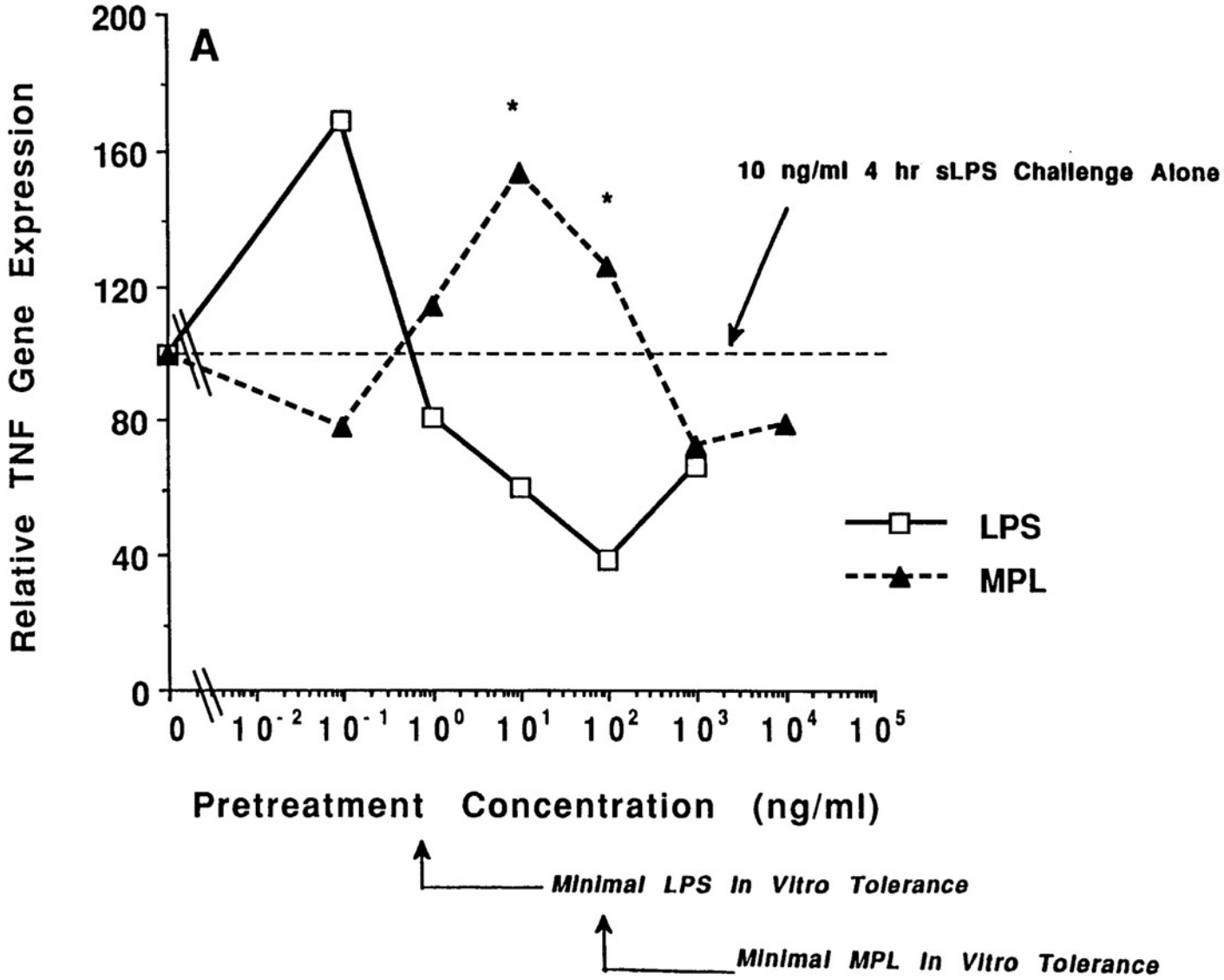
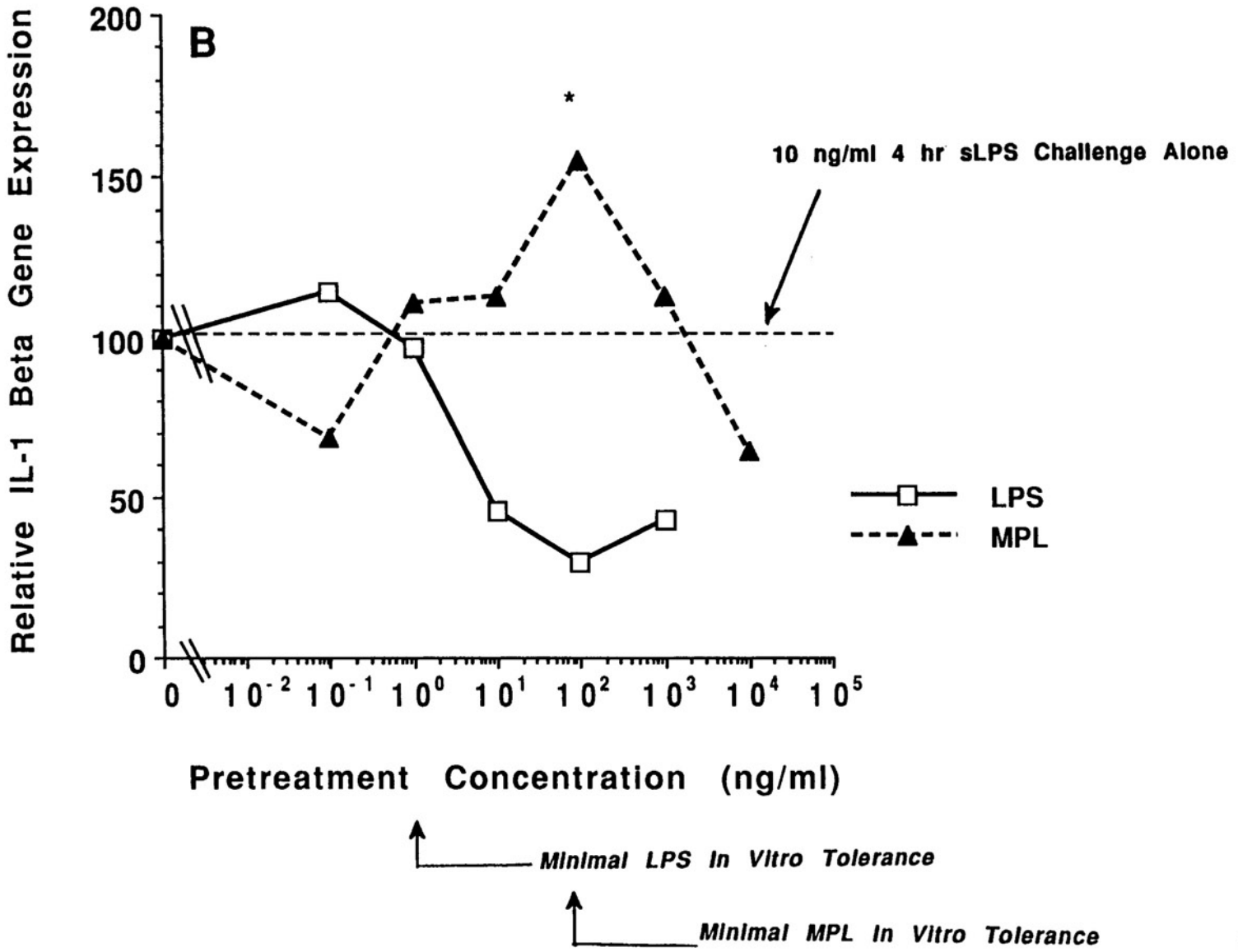
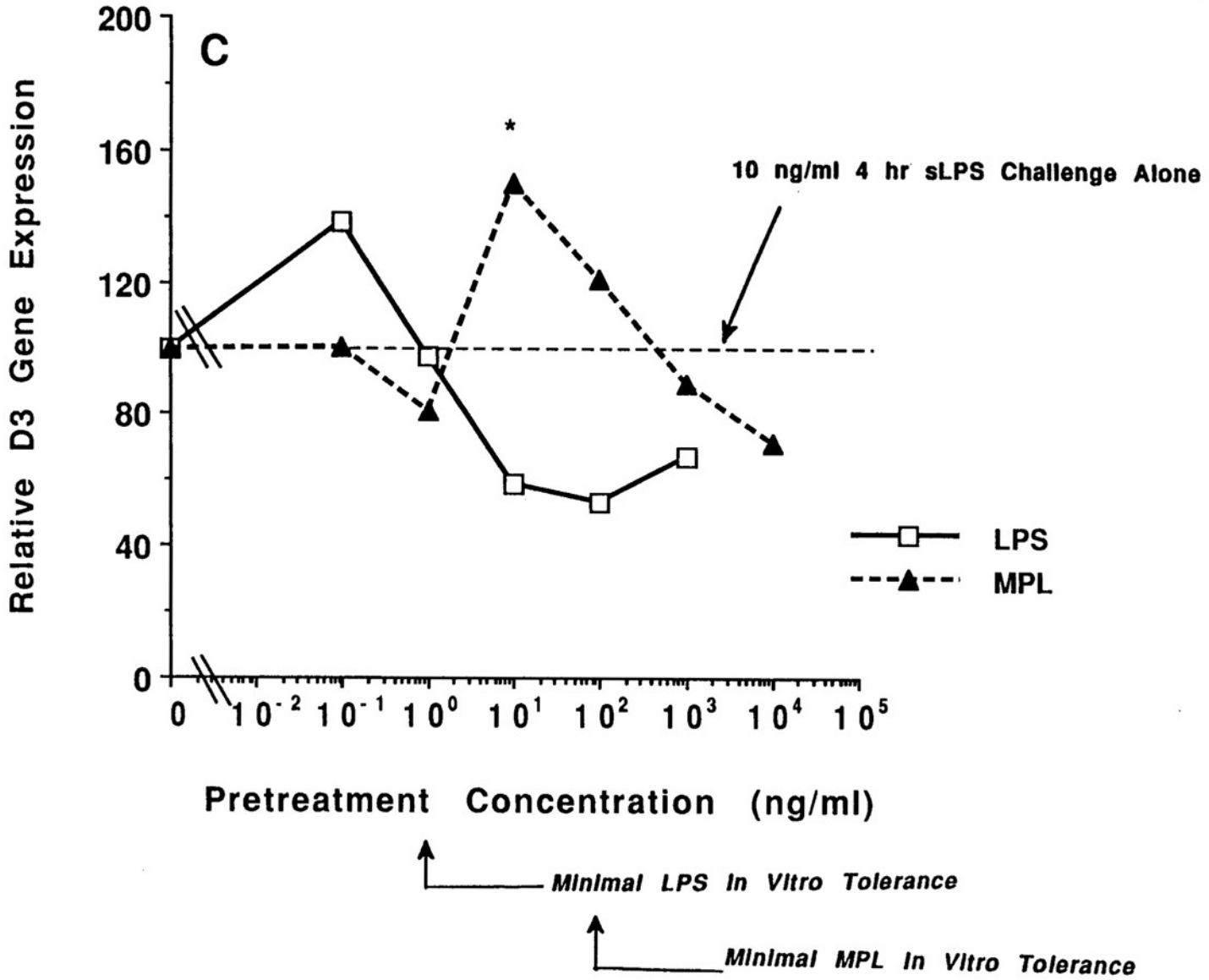

B
Relative D7 Gene Expression
120
100
80
60
40
20
0
2
4
6
8
10
12
Time in Hours
LPS
MPL

Figure 33. Comparison of TNFα (Figure 33 A), IL-1β (Figure 33 B), and D3 (Figure 33 C) steady-state mRNA expression in macrophages pretreated with LPS or MPL. Macrophages were stimulated with the indicated concentrations of LPS or MPL for 20 hours and then "challenged" with LPS (10 ng/ml) for 4 hours, as described in the Materials and Methods. Results represent PhosphorImager and scanning densitometer data measurements of total RNA on Northern blots from 4 separate experiments. Relative gene expression has been normalized to the expression of β-actin by dividing the volume measurement for each gene by the volume measurement for β-actin in the same lane (gene/β–actin ratio). Measurements were then normalized to the maximal expression induced by the 10 ng/ml *E. coli* K235 LPS (100%) challenge stimulation of medium-pretreated cells (dotted line), by dividing the gene/β-actin ratio for each treatment by the gene/β-actin ratio for the 10 ng/ml challenge, as described in the Materials and Methods. The graphed values are the computer generated Least Squares Means. Asterisks indicate expression values significantly different ($p < 0.0043$) from LPS-induced expression at 4 hours after stimulation, as determined by statistical analysis of Least Squared Means comparison of the compiled log transformed data. Data were analysed by Mr. Jian-Zheng Zhou of the University of Maryland Department of Animal Science and Dr. Lawrence Douglass of the University of Maryland Department of Statistics using IBM format SAS software. Arrows under the X-axis indicate the LPS and MPL concentrations for which minimal *in vitro* tolerance was observed, as assessed by decreased secreted TNF.


A
200
160
120
80
40
0
Relative TNF Gene Expression
*
*
10 ng/ml 4 hr sLPS Challenge Alone
LPS
MPL
0 10-2 10-1 10 0 10 1 10 2 10 3 10 4 10 5
Pretreatment Concentration (ng/ml)
Minimal LPS In Vitro Tolerance
Minimal MPL In Vitro Tolerance


B
Relative IL-1 Beta Gene Expression
200
150
100
50
0
*
10 ng/ml 4 hr sLPS Challenge Alone
LPS
MPL
0 10-2 10-1 10 0 10 1 10 2 10 3 10 4 10 5
Pretreatment Concentration (ng/ml)
Minimal LPS In Vitro Tolerance
Minimal MPL In Vitro Tolerance

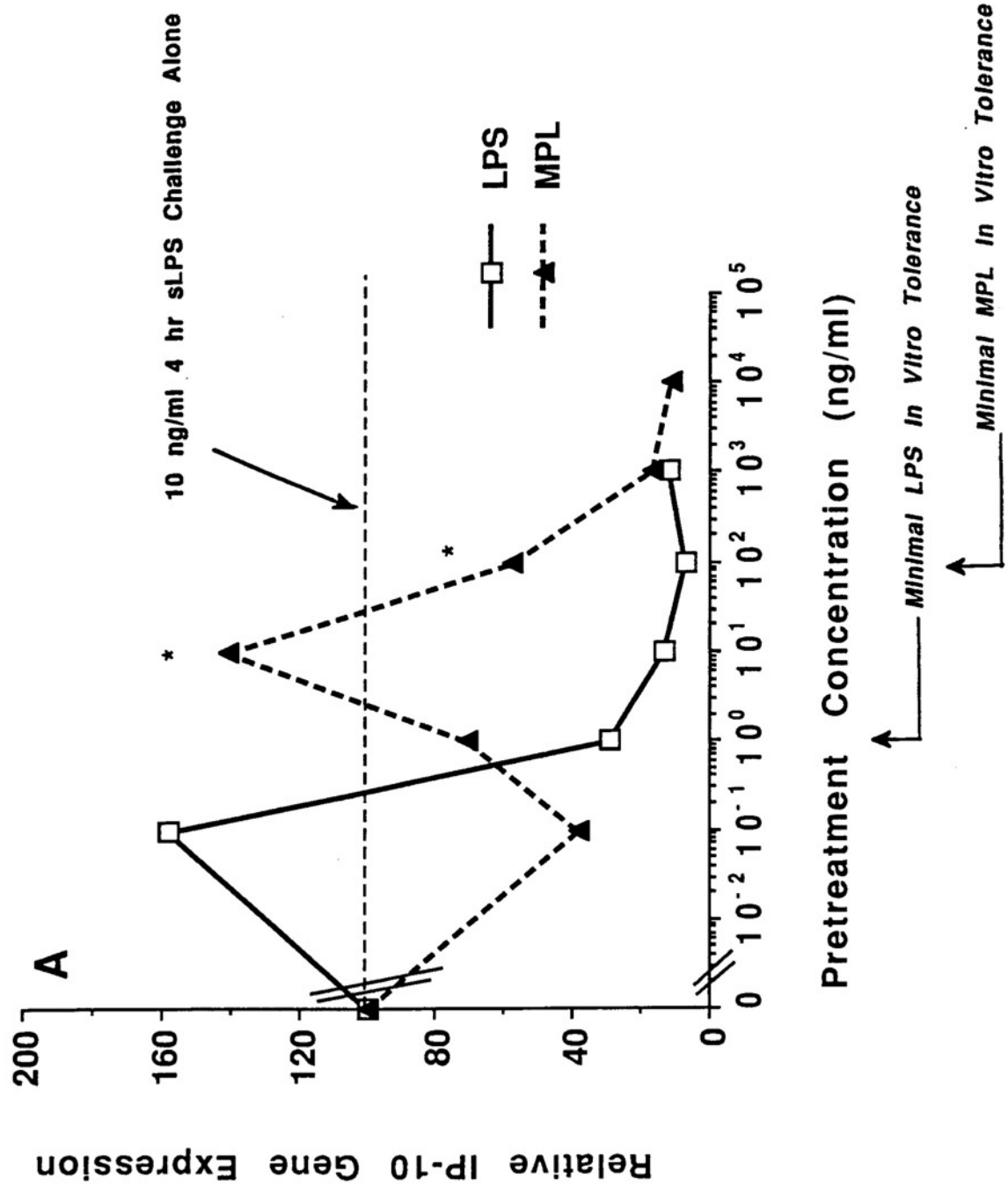

C
200
160
120
80
40
0
*
10 ng/ml 4 hr sLPS Challenge Alone
LPS
MPL
Relative D3 Gene Expression
0 10-2 10-1 100 101 102 103 104 105
Pretreatment Concentration (ng/ml)
Minimal LPS In Vitro Tolerance
Minimal MPL In Vitro Tolerance

suppressed slightly upon "challenge" when cells were exposed to a very low pretreatment concentration of MPL (< 1 ng/ml). However, upon exposure to moderate concentrations (1 - 100 ng/ml) shown previously to induce tolerance *in vitro*, LPS-pretreated cells responded to challenge with a significant decrease in gene expression (e.g., 40-60% of control levels), whereas in MPL- pretreated cells exposed to moderate (1 - 100 ng/ml) pretreatment, levels of expression were significantly superinduced. MPL-induced TNF, IL-1, and D3 were suppressed only at $\geq$ 1,000 ng/ml pretreatment.

In contrast to the moderate suppression of TNFα, IL-1β, and D3 expression after "challenge" that was induced by pretreatment of macrophages with LPS and MPL, post-challenge IP-10 and D8 gene expression was reduced profoundly (to near background levels) by pretreatment with tolerance-inducing concentrations of LPS or MPL (Figure 34 A, B). At less than tolerance-inducing concentrations of either LPS and MPL, IP-10 and D8 mRNA expression were superinduced similarly to TNFα, IL-1β, and D3. At very low concentrations of MPL pretreatment, gene expression was again moderately suppressed.

For genes D2 (Figure 35 A) and D7 (data not shown) there was no significant suppression of steady-state mRNA expression upon LPS challenge of either LPS- or MPL-pretreated macrophages. Pretreatment resulted in only a modest superinduction of D2 and D7 mRNA expression, (e.g., above the level induced by the 10 ng/ml LPS "challenge" of medium-pretreated cultures), and no statistical differences were observed between LPS- and MPL-pretreated cultures for these two genes. When compared directly to LPS-induced IP-10 expression, which was strongly suppressed by LPS pretreatment (Figure 34 A), both D2 and D7 expression were maximal at concentrations of LPS pretreatment that completely suppressed IP-10. This relationship is illustrated in the composite auto-radiograph shown in Figure 36.

Figure 34. Comparison of IP-10 (Figure 34 A) and D8 (Figure 34 B) steady-state mRNA expression in macrophages pretreated with LPS or MPL. Macrophages were stimulated with the indicated concentrations of LPS or MPL for 20 hours and then "challenged" with LPS (10 ng/ml) for 4 hours, as described in the Materials and Methods. Results represent PhosphorImager and scanning densitometer data measurements of total RNA on Northern blots from 4 separate experiments. Relative gene expression has been normalized to the expression of β-actin by dividing the volume measurement for each gene by the volume measurement for β-actin in the same lane (gene/β–actin ratio). Measurements were then normalized to the maximal expression induced by the 10 ng/ml *E. coli* K235 LPS (100%) challenge stimulation of medium-pretreated cells (dotted line), by dividing the gene/β-actin ratio for each treatment by the gene/β-actin ratio for the 10 ng/ml challenge, as described in the Materials and Methods. The graphed values are the computer generated Least Squares Means. Asterisks indicate expression after 4 hours after stimulation values significantly different ($p < 0.0043$) from LPS-induced expression at 4 hours after stimulation, as determined by statistical analysis of Least Squared Means comparison of the compiled log transformed data. Data were analysed by Mr. Jian-Zheng Zhou of the University of Maryland Department of Animal Science and Dr. Lawrence Douglass of the University of Maryland Department of Statistics using IBM format SAS software. Arrows under the X-axis indicate the LPS and MPL concentrations for which minimal *in vitro* tolerance was observed, as assessed by decreased secreted TNF activity.


A
Relative IP-10 Gene Expression
200
160
120
80
40
0
10 ng/ml 4 hr sLPS Challenge Alone
*
*
LPS
MPL
0
10^{-2} 10^{-1} 10^{0} 10^{1} 10^{2} 10^{3} 10^{4} 10^{5}
Pretreatment Concentration (ng/ml)
Minimal LPS In Vitro Tolerance
Minimal MPL In Vitro Tolerance

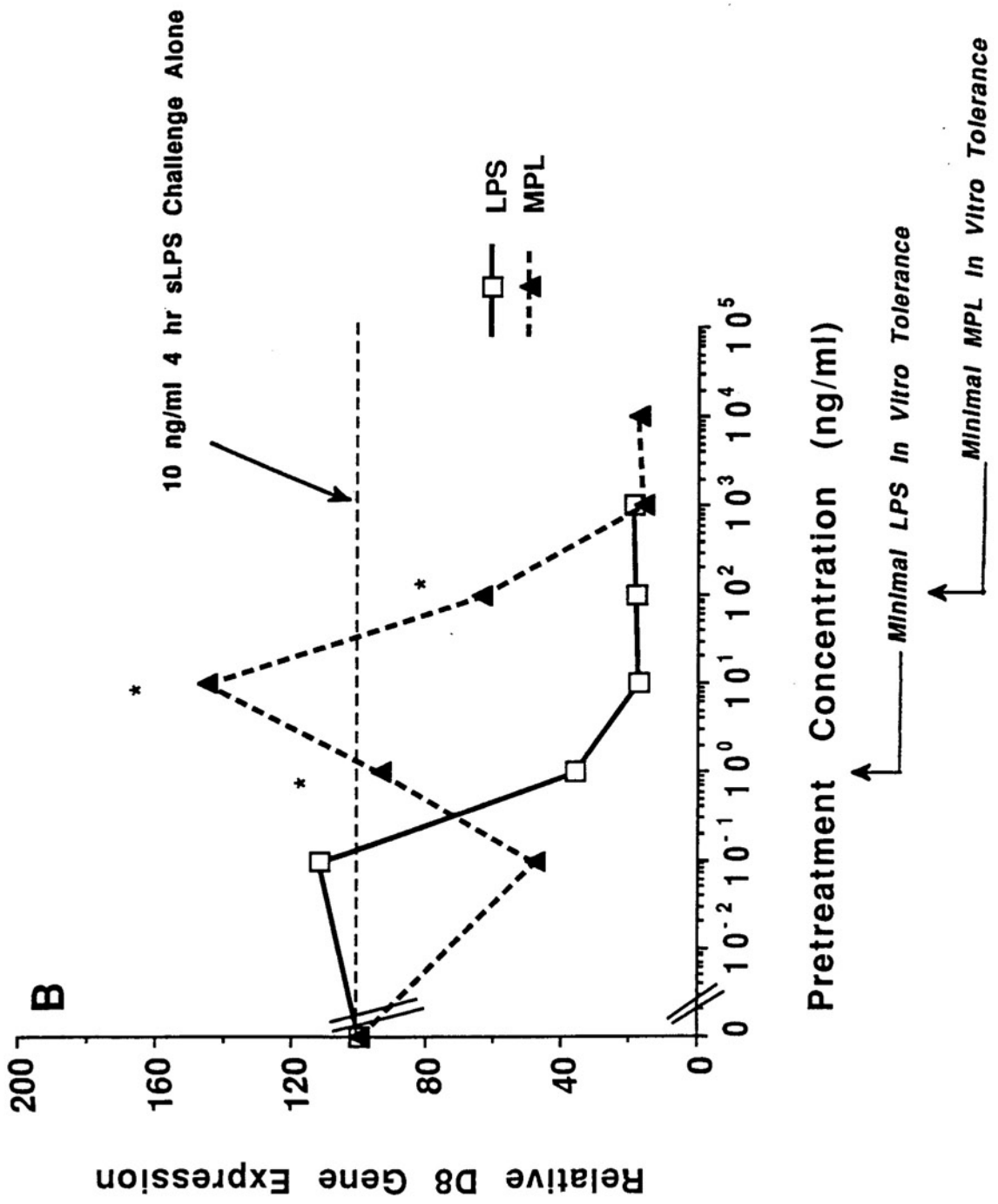

B
Relative D8 Gene Expression
200
160
120
80
40
0
10 ng/ml 4 hr sLPS Challenge Alone
LPS
MPL
*
*
*
0
10^-2 10^-1 10^0 10^1 10^2 10^3 10^4 10^5
Pretreatment Concentration (ng/ml)
Minimal LPS In Vitro Tolerance
Minimal MPL In Vitro Tolerance

Figure 35. Comparison of D2 (Figure 35 A) steady-state mRNA expression in macrophages pretreated with LPS or MPL. Macrophages were stimulated with the indicated concentrations of LPS or MPL for 20 hours and then "challenged" with LPS (10 ng/ml) for 4 hours, as described in the Materials and Methods. Results represent PhosphorImager and scanning densitometer data measurements of total RNA on Northern blots from 4 separate experiments. Relative gene expression has been normalized to the expression of β-actin by dividing the volume measurement for each gene by the volume measurement for β-actin in the same lane (gene/β–actin ratio). Measurements were then normalized to the maximal expression induced by the 10 ng/ml *E. coli* K235 LPS (100%) challenge stimulation of medium-pretreated cells (dotted line), by dividing the gene/β-actin ratio for each treatment by the gene/β-actin ratio for the 10 ng/ml challenge, as described in the Materials and Methods. The graphed values are the computer generated Least Squares Means. Asterisks indicate expression after 4 hours after stimulation values significantly different from LPS-induced expression, as determined by statistical analysis of Least Squared Means comparison of the compiled log transformed data. Data were analysed by Mr. Jian-Zheng Zhou of the University of Maryland Department of Animal Science and Dr. Lawrence Douglass of the University of Maryland Department of Statistics using IBM format SAS software. Arrows under the X-axis indicate the LPS and MPL concentrations for which minimal *in vitro* tolerance was observed, as assessed by decreased secreted TNF activity.

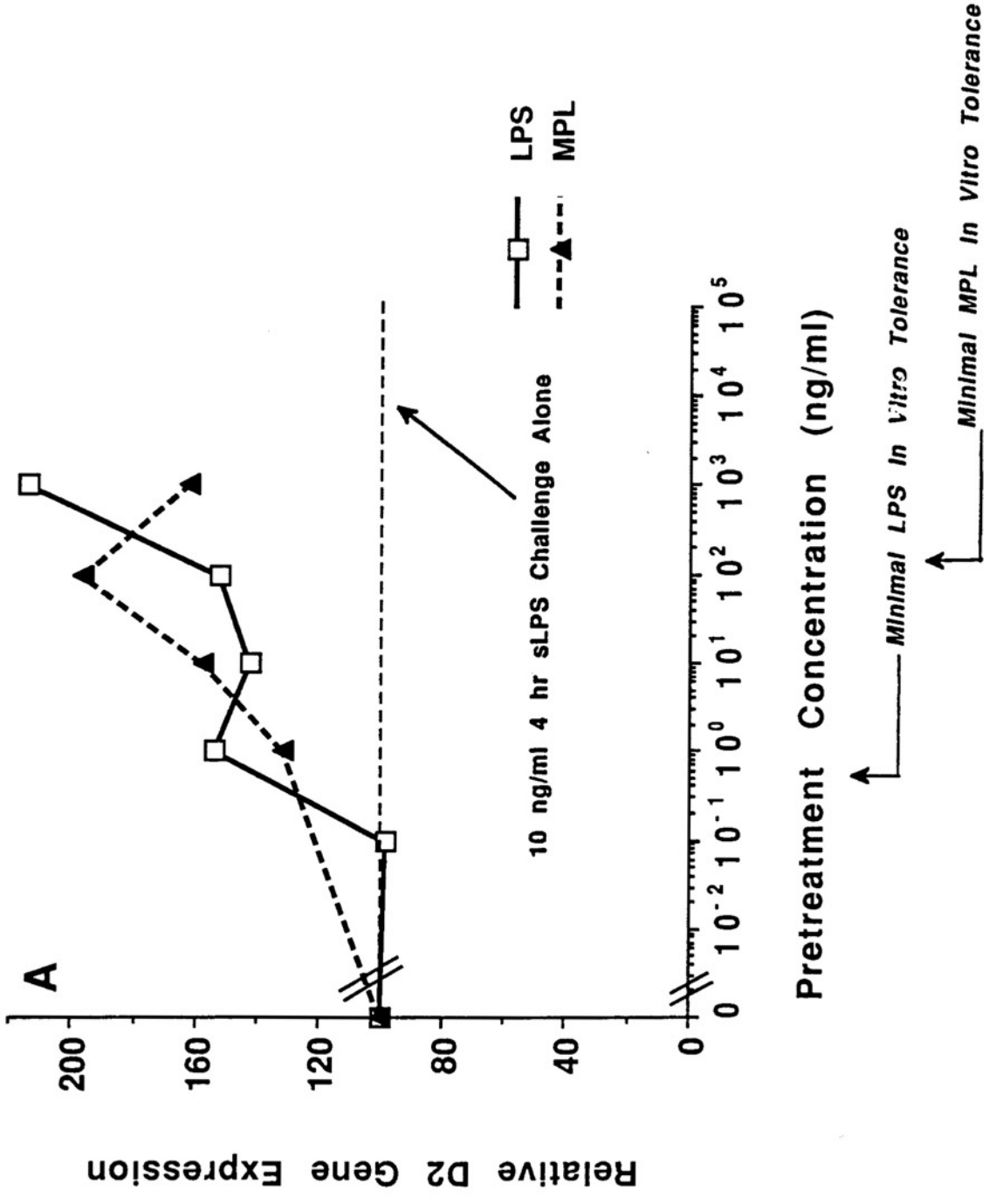
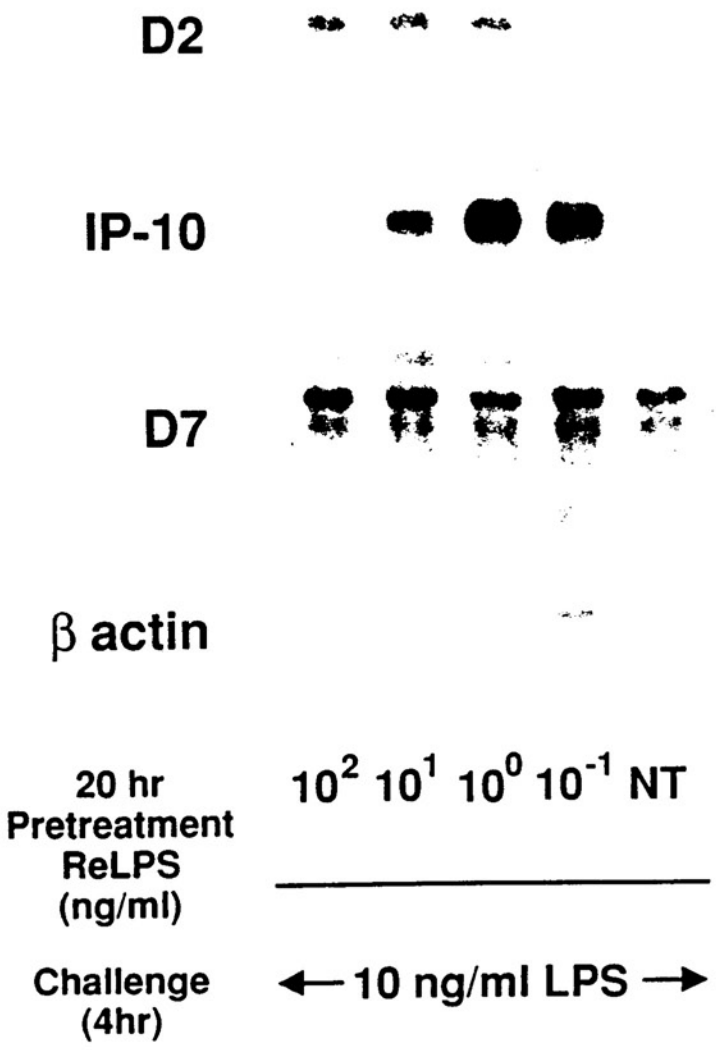

A
Relative D2 Gene Expression
200
160
120
80
40
0
10 ng/ml 4 hr sLPS Challenge Alone
LPS
MPL
0 10-2 10-1 100 101 102 103 104 105
Pretreatment Concentration (ng/ml)
Minimal LPS In Vitro Tolerance
Minimal MPL In Vitro Tolerance

Figure 36 Composite autoradiographs of Northern blots from a single representative experiment depicting D2, IP-10, and D7 expression after 4 hours of LPS "challenge" (10 ng/ml *E. coli* K235 LPS). Macrophages were pretreated with the indicated concentrations of ReLPS. LPS, MPL and DPLA for 20 hours. The expression of β-actin steady-state mRNA in the same lanes is shown for comparative purposes.


D2
IP-10
D7
β actin
20 hr Pretreatment ReLPS (ng/ml)
10^2 10^1 10^0 10^{-1} NT
Challenge (4hr)
← 10 ng/ml LPS →

DISCUSSION

Our understanding of the mechanisms which underlie endotoxic shock has grown immensely due to discovery of the many cytokines induced by LPS and elucidation of their functions. In particular, macrophage-derived monokines are felt to contribute significantly to the many physiological alterations attributed to endotoxin administration (reviewed in Vogel and Hogan, 1990). Within the first several hours after injection of LPS, macrophage-secreted TNF (cachectin) (Beutler *et al.*, 1985; Beutler and Cerami, 1987), IL-1 (Knudsen *et al.*, 1986; Matsushima *et al.*, 1986), and IFN-α/β have peaked or are approaching peak concentrations in the serum. At four to six hours after injection of LPS, CSF, IL-6, and Glucocorticoid Antagonizing Factor (GAF), have been shown to rise to maximum levels (Williams *et al.*, 1983; Hill *et al.*, 1986; Metcalf, 1971; Moore *et al.*, 1976; 1978; 1985).

It is now recognized that many of these cytokines serve multiple functions, and when administered to animals, they mimic the responses induced by LPS (Bauss *et al.*, 1987; Vogel *et al.*, 1987, 1988). In addition, it is well-established that structurally unrelated cytokines often exhibit overlapping activities. For example, fever is a classic symptom associated with LPS administration. Administration of any of three distinct cytokines, e.g. IL-1, TNF, or IFN, leads to prostaglandin-mediated fever similar to that seen in response to LPS. In addition, a high concentration of recombinant TNF causes two temperature spikes: the first, which is TNF-induced and a later one which is due

to the action of TNF-stimulated IL-1 (Dinarello *et al.*, 1986). Collectively, these factors are referred to as "endogenous pyrogens." Thus, multiple factors induced by LPS can potentially contribute to the elicitation of a given biological response.

LPS also induces a macrophage-derived soluble factor(s) referred to as Glucocorticoid Antagonizing Factor (GAF) (Moore *et al.*, 1976,1985). GAF has been shown to cause hypoglycemia by blocking the induction of PEPCK (phosphoenolpyruvate carboxykinase) mRNA by LPS-induced glucocorticoids (Goodrum and Berry, 1979; Moore *et al.*, 1985; Hill *et al.*, 1986). It is the enzymatic action of PEPCK that governs the conversion of oxaloacetate to phosphoenolpyruvate, which is the rate-limiting step in gluconeogenesis. Therefore, GAF induces hypoglycemia by antagonizing the replacement of lost serum glucose via gluconeogenesis. Pyruvate kinase activity in the liver is also increased by exposure to endotoxin, leading to a rapid utilization of stored glycogen and further depletion of the body's glucose stores (Snyder *et al.*, 1971).

Another phenomenon induced by LPS, and mimicked by cytokine administration, is a transient state of hyporesponsiveness which is called "early endotoxin tolerance" (Favorite and Morgan, 1942). The phenomenon is not transferrable with serum (Greisman and Woodward, 1965), but reticuloendothelial blockade inhibits tolerance induction (Beeson, 1946). Macrophages from LPS-tolerant mice produced reduced levels of endogenous pyrogen and prostaglandin E_2, respectively, when stimulated *in vitro* by LPS (Dinarello *et al.*, 1968, Dinarello and Bernheim, 1981). Thus, failure of "tolerant" macrophages to produce those soluble factors responsible for endotoxicity was proposed to be the cellular basis for "early endotoxin tolerance."

This notion was extended *in vivo* by Williams *et al.* (1983) who showed that in mice rendered tolerant, the level of endotoxin hyporesponsiveness paralleled serum CSF levels inducible upon LPS "challenge." Madonna and Vogel (1985) confirmed these results and showed that alterations in LPS-induced CSF and LD_{50} correlated with changes in bone marrow cell size and changes in numbers of macrophage progenitors (Madonna and Vogel, 1985). Subsequent studies showed that both toxicity and a state of LPS hyporesponsiveness could be induced by the highly dose-dependent synergistic activity of recombinant IL-1α and TNFα, and that the doses which resulted in "tolerance" also led to the hematopoeitic changes seen in response to tolerizing doses of either LPS or MPL (Vogel *et al.*, 1988).

In 1984, Ribi and his colleagues characterized a non-toxic derivative of LPS called monophosphoryl lipid A (MPL). Madonna *et al.* (1986) showed that MPL could also induce early endotoxin tolerance. In those experiments, tolerance was assessed on the basis of inhibition of LPS-induced CSF and IFN production, as well as by a significant increase in LD_{50}. The purposes of the *in vivo* experiments presented herein were: (i) to study the effects of early endotoxin tolerance on an additional LPS toxic manifestation, e.g., hypoglycemia, and to measure the effect of tolerance induction on the secretion of other cytokines, including TNF, IL-1 and IL-6; (ii) to compare the relative cytokine-inducing capacities of equivalent tolerance-inducing doses of LPS and MPL; and, (iii) to assess the roles of two specific cytokines, e.g., IL-1 and TNF, in the mediation of early endotoxin tolerance *in vivo*.

When LPS was compared to MPL for tolerance-inducing ability, the original observations of Madonna *et al.* (1986) were confirmed and extended in

inbred mice. We found that the tolerance-inducing capacity of 25 μg of LPS could be reproduced by administration of 200 μg of MPL, as assessed by LPS-induced CSF production (Figure 3), hypoglycemia (Figure 4), IL-6 (Table I) and IL-1 production (Figure 8). This same dose of MPL, however, was somewhat less "tolerogenic" than LPS with regard to IFN and TNF production (Figures 5 and 6). Nonetheless, administration of MPL resulted in "early endotoxin tolerance," as evidenced by a significant decrease in both the TNF and IFN levels evoked upon LPS challenge injection (Figures 6 and 7).

Both ReLPS (containing KDO_2-lipid A), and diphosphoryl lipid A (DPLA) also induced tolerance *in vivo* comparably to 25 μg LPS with respect to CSF induction. However, unlike MPL, only 25 μg of either derivative was completely efficacious. Induction of equivalent tolerance by ReLPS and DPLA indicates that the dose differential between LPS and MPL is not solely a difference due to activity of the polysaccharide portion of smooth LPS or due to a large disparity in the molar ratios of the substances. Thus, the large dose necessary for MPL to induce tolerance appears to be related to the lack of the position 1 phosphoryl group, since neither ReLPS nor DPLA differs significantly from MPL in size (Figures 2 and 18). In 1987, Reitschel *et al.* tested the relative toxicity of various forms of MPL *versus E. coli* lipid A. Results suggested that the additional fatty acid on the heptaacyl portion of the *S. minnesota* MPL derivative (which represents ~20% of the total MPL preparation; Nilofer Qureshi, personal communication), as well as the absence of the hydrophilic phosphoryl group, disrupted the conformation of the toxic moiety and reduced its biological activity. However, like the *S. minnesota* MPL, 200 μg of synthetic hexaacyl *E. coli* MPL was found to induce tolerance equivalently to 25 μg LPS, showing that the proportion of heptaacyl lipid A in the *S. minnesota* MPL is not responsible for its

diminished tolerance-inducing ability (Table II), but that removal of the phosphoryl group solely results in the necessity of a higher dose to induce equivalent tolerance (Figure 3).

One physical consequence of the removal of the phosphoryl group has been studied. A decrease in hydrophilicity (solubility) and an increase in phase transition temperature (Rietschel *et al.*,1987) was demonstrated to be caused by removal of this highly charged moiety. Increased hydrophobicity may cause profound changes in the way MPL is perceived by host cells. Since it is more hydrophobic, MPL may tend to form larger micelles than LPS in physiologic solutions. In fact, electron microscopy has shown that MPL, even though sonicated and treated to increase solubility, reverts to larger aggregates in the presence of SRBC stroma than does LPS (Figure 16). Thus, the effective concentration of MPL available to interact with cell membranes would be reduced significantly and its relative lack of bioavailability would be reflected in the higher dose of MPL than LPS necessary to induce tolerance. This fact alone would reduce the toxicity of MPL relative to LPS since part of the toxicity associated with LPS has been attributed to direct intercalation of the hydrophobic fatty acids of LPS into host cell membranes, an interaction which can lead to dissolution and rupture of nuclear, plasma, golgi, and mitochondrial membranes (Kang *et al.*, 1990). The resultant disruption of membrane integrity destroys mitochondrial compartmentalization necessary to maintain proton motive force, disrupts golgi processing of synthesized proteins, and separation of the nuclear and cytoplasmic compartments. Any decrease in the interaction of MPL with membranes would, therefore, make it less toxic in general. Future studies using electron microscopy should address the interaction of LPS and DPLA *versus* MPL with macrophage plasma membranes.

An intriguing finding was the observation that 25 μg of LPS and 200 μg of MPL resulted in comparable levels of CSF following initial injection (Figure 9), whereas levels of IFN, TNF, and IL-6 induced were approximately ten-fold less in the MPL-injected mice (Tables III and IV, Figure 10). Although not 10-fold less in protein concentration, circulating levels of IL-1α induced by MPL were also clearly lower than those induced by LPS (Figure 11). It is important to note that IL-1α/β, IFN-α/β, TNFα, and IL-6 have all been shown to be macrophage-derived, while the cellular origin of LPS-induced CSF has not been clearly delineated. Our findings using the rIL-1ra have shed light on this problem. Since (i) rIL-1ra binds preferentially to the murine "Type 1" IL-1 receptors, which are found predominantly on fibroblasts and T cells, but not to mouse "Type 2" receptors, that are expressed on macrophages and B cells (Bomsztyk *et al.*, 1989; Hannum *et al.*, 1990), and (ii) athymic nude mice respond normally to LPS to produce CSF (Madonna and Vogel, 1986), the data imply that LPS-induced CSF is predominantly of fibroblast, and not macrophage, origin. Indeed, this hypothesis is consistent with the finding that LPS- and MPL-induced CSF is approximately equivalent and that the levels of macrophage-derived cytokines induced by LPS *versus* MPL are widely disparate. Previous studies have demonstrated that both IL-1 and TNF stimulate fibroblasts *in vitro* to produce CSF (Munker *et al.*, 1986; Zucali *et al.*, 1986), and that the *in vivo* production of IL-1 and TNF after administration of LPS is a very early response (Cannon *et al.*, 1990; Hannum *et al.*, 1990) which precedes the appearance of LPS-induced CSF (Henricson *et al.*, 1990; reviewed in Vogel and Hogan 1990).

Since CSF was the only cytokine found to be stimulated equivalently by LPS and MPL, one possibility is that tolerance is mediated solely through the action of CSF. Alternatively, it is possible that tolerance is induced by a

substance not measured in the experiments (like the IL-1 receptor antagonist). Synergy between TNF, IL-1, and IL-6 is responsible for enhancement of a wide variety of LPS-inducible responses (Neta *et al.*, 1992). Thus, early endotoxin tolerance may be the result of some non-toxic combination of cytokines that are elicited by MPL as well as LPS. This latter possibility is consistent with the synergistic toxicity observed between recombinant IL-1α and TNFα *in vivo*, whereas lower dose combinations of these two cytokines result in a "tolerant" state. Thus, the diminished toxicity of MPL when compared to LPS may be due to its induction of lower levels of toxic intermediaries such as TNF, IL-1 and/or IL-6 upon initial injection, and yet enough may be produced for the synergy required to induce tolerance. Since an ELISA was used to measure IL-1α, the relationship between immunoreactive IL-1α protein and bioactivity is unknown in this study. A lower level of IL-1α might actually translate into slightly less bioactivity, or even significantly less, if net IL-1 activity ultimately depends on other factors such as LPS- or MPL-induced enzymatic cleavage of a larger precursor form of IL-1 (Cerretti *et al.*, 1992), or the presence of currently unrecognized MPL-induced inhibitory factors. The fact that IL-1α is largely cell-associated adds an additional caveat against using immunoreactive protein levels to assess activity. However, when a specific assay for murine IL-1β becomes available, it would be desirable to measure its presence in sera of LPS-*versus* MPL-treated mice.

The results derived from experiments in which rIL-1ra and anti-TNF antibody were used as specific cytokine antagonists show that both IL-1 and TNF appear to act as intermediates in induction of both CSF and early endotoxin tolerance induced by LPS. Incomplete inhibition of CSF activity and induction of tolerance was observed, even when the cytokine antagonists were administered

together. This may be due, in part, to direct competition of the IL-1ra and IL-1 for available binding sites, since only a low percentage of IL-1 receptors need to be occupied for signal transduction (Arend *et al.*, 1990). Alternately, IL-1 may induce its effects on CSF or tolerance induction via the IL-1 "Type 2" receptor which is not blocked by rIL-1ra in the mouse (Bomsztyk *et al.*, 1989; Hannum *et al.*, 1990). The incomplete inhibition of LPS-induced CSF by the two specific cytokine antagonists may be attributable to factors other than IL-1 and TNF which act independently to induce CSF. Although synergy between IL-1 and TNF has been repeatedly demonstrated (Neta *et al.*, 1991), this was not observed for induction of CSF *in vivo* using recombinant IL-1 and TNF (Vogel *et al.*, 1987). The incomplete ablation of LPS-induced CSF by rIL-1ra alone is not surprising in view of the fact that rIL-1ra had no effect on LPS-induced TNF production (data not shown). Since rTNFα has also been shown to induce CSF activity *in vivo* (Vogel *et al.*, 1987), some portion of the LPS-induced CSF activity may be due to LPS-induced TNF that, in turn, induces CSF. Administration of anti-TNF antibody to mice also reduced LPS-induced CSF by approximately 50%, but no more (Vogel and Havell, 1990); an indirect indication that TNF is participatory, but not singular, in the pathway by which LPS induces CSF.

The rIL-1ra and anti-TNF antibody did not have any activity in the bone marrow colony assay, indicating that they do not antagonize CSF activity *in vitro* by binding CSF or by inhibiting the action of IL-1 or TNF generated by cells present during the assay. That inhibition of LPS-induced CSF by rIL-1ra is observed, even 3 days following administration of rIL-1ra, suggests that rIL-1ra is neither degraded nor eliminated immediately from circulation. Since rIL-1ra "carry over" may dampen LPS-induced CSF production on Day 3, the observed increase in CSF production in tolerized animals "challenged" with LPS may

actually be even more dramatic than indicated (Figure 13). The finding that rIL-1ra partially reverses the induction of early endotoxin tolerance is consistent with previous work which demonstrated that LPS-induced early endotoxin tolerance could be simulated by injection of both rIL-1α and rTNFα; neither alone induced tolerance (Vogel *et al.*, 1988). Thus, the rIL-1ra and anti-TNF antibody appear to block LPS-induced IL-1 and TNF, respectively, from contributing to the cytokine synergy required for elicitation of early endotoxin tolerance. Collectively, the results support the position that IL-1 and TNF are indeed intermediates in the pathway for LPS-induced CSF and illustrates the requirement of IL-1 and TNF for the initiation of early endotoxin tolerance by LPS *in vivo*.

LPS-induced hypoglycemia is mediated by a variety of mechanisms that have also been ascribed to the action of LPS-induced cytokines. First, LPS stimulates glucose consumption by monocytic phagocytes and proliferating B and T cell populations, as well as other tissues (Smith *et al.*, 1983). Injection of recombinant cytokines has been used to argue that cytokines are indeed responsible for LPS' toxic symptoms *in vivo*. DelRey and Besedofsky (1987, 1989) determined that upon injection, recombinant IL-1α initially caused hyperglycemia in rats and subsequently caused hypoglycemia which was paralleled by an increase in glucagon and corticosterone. Both recombinant IL-1α and recombinant TNFα have been shown to induce hypoglycemia *in vivo* (Bauss *et al.*, 1987; Del Rey and Besedofsky, 1989). By itself, TNF injection causes hypoglycemia (Tracey *et al.*, 1986), but anti-TNF antibody has been shown unable to mitigate LPS-induced hypoglycemia (Vogel and Havell, 1990). LPS-induced reduction in serum glucose must, therefore, involve biochemical pathways that do not depend solely on TNF's ability to induce IL-1 (Dinarello, 1986). TNF has also been found to increase the rate of glucose consumption in tissues, and

initiates hyperinsulinemia (Yelich *et al.*, 1987; Sacco-Gibson *et al.*, 1988). Together, IL-1 and TNF augment lactate levels by upregulating the rate of glycolysis that is facilitated by an IL-1-induced increase in hexose transport molecules (Bird *et al.*, 1990).

In the studies shown here, IL-1 was demonstrated to be a direct intermediate in the production of LPS-induced hypoglycemia, since 300 μg of rIL-1ra was able to reverse LPS-induced hypoglycemia significantly, but not completely. This result is consistent with the knowledge that multiple pathways are involved in the generation of hypoglycemia. Although rIL-1ra does not change the glucose level when injected alone, its administration three days prior to LPS "challenge" reverses LPS-induced hypoglycemia to a level equivalent to that found in tolerized animals (Compare Table V, E and F). This reversal of LPS' toxic effects suggests the possibility that tolerance may be based upon the production and action of a number of inhibitory factors, like rIL-1ra, after initial exposure to LPS, which competetively inhibit toxic symptom-inducing cytokines upon re-exposure. Although the rIL-1ra is cleared from the circulation rapidly, it may act at a time remote from its existence by causing internalization of cytokine receptors, or it may serve an intracellular inhibitory function that is as yet undefined. It is also interesting to note that while the "carryover" effect of rIL-1ra the third day after injection was minimal with respect to CSF induction, it was quite significant ($p= 0.003$) with respect to LPS-elicited hypoglycemia. This difference may reflect differential dependencies on IL-1 for the induction of CSF *versus* hypoglycemia, or differential expression of "Type 1" receptors by the cells of the target organs.

Although rIL-1ra has been proposed for use in the treatment of inflammatory diseases which involve aberrant production of IL-1, such as

rheumatoid arthritis (1), its application may also be extended to cases of severe systemic Gram negative infection, burn injury, or trauma where initiation of tolerance may hamper the adequate immune response of the individual to produce CSF (Loose and Turinsky, 1979; Parillo, 1990; Peterson *et al.*, 1983; Peterson *et al.*, 1985), or it may be used initially to ameliorate symptoms of sepsis-associated hypoglycemia. Clinical trials utilizing rIL-1ra in patients with septic shock are currently underway.

Although the electron microscopic analysis presented in Figure 16 suggests that MPL forms larger micellar structures with reduced capacity for membrane contact, it is also possible that MPL has lowered toxicity due to inadequate interaction with specific LPS receptors as a result of its relative hydrophobicity, or that a different three dimensional fatty acid conformation (caused by the removal of the phosphoryl group) makes MPL interact with specific lipid A binding proteins with lower binding capacity. The conditions of high concentration required to visualize micelles by electron microscopy are unlikely to be achieved under conditions of clinical sepsis. Moreover, there is recent ample evidence for the existence of specific lipid A receptors on macrophages (reviewed in Morrison, 1990). Two lines of evidence presented in this study suggest that LPS and MPL interact differentially with specific lipid A receptors on the surface of macrophages. Although this phenomenon could be accounted for solely by the larger micellar structure in MPL, it is likely that at much lower concentrations (e.g., ng/ml) the capacity of LPS *versus* MPL monomers or small aggregates to interact with specific receptors may depend largely on their binding capacity for the LPS-binding proteins in the macrophage membranes.

However, quantitative analysis of receptor-ligand interactions of LPS with specific cell surface proteins has proven difficult or impossible because of non-specific binding and insertion of LPS directly into cell membrane lipid bilayers at high concentration. The problems associated with this type of receptor-ligand analysis are further compounded by the fact that there are multiple LPS receptors, none of which have been characterized for their relative expression, strengths of binding, or importance in intracellular signalling. However, the ~80 kDa lipid A receptor, when engaged by a specific monoclonal antibody, delivers a signal to Lps^n, but not Lps^d, macrophages to induce TNF, tumor cytotoxicity, and NO synthesis. Results from the competitive inhibition of Rα5D3 anti-lipid A receptor antibody (Figure 17) suggest that MPL has a lower binding capacity for the ~80 kDa receptor than LPS. This conclusion is based on the finding that significantly more MPL than LPS is required to inhibit binding of the anti-receptor antibody to macrophages. However, there were multiple concerns about this type of indirect analysis. These include steric hinderance on the part of the polysaccharide, as well as the steric constraints implicit in bivalent binding of antibody to the cell surface. Therefore, this approach was abandoned and an alternate one adopted.

To circumvent these problems, a competitive inhibitor of lipid A-cell interaction has been used to compare structure to function relationships in the interaction of sLPS and its derivatives with macrophage receptor(s). Recent studies have shown that the lipid A derived from *R. sphaeroides* fails to induce bioactivity and yet acts as a competitive inhibitor of LPS- or lipid A-induced phenomena (Salimath, *et al.*, 1983; Strittmatter *et al.*, 1983; Rietschel *et al.*, 1987; Qureshi *et al.*, 1991b). Structural analysis of this molecule has led to the hypothesis that within the pentaaclyated RsDPLA, the short length of the fatty

acids at R1 and R3, and an unsaturated acyloxyacyl group linked at R2 contribute to its lack of toxicity. (Qureshi *et al.*, 1991a; Rietschel *et al.*, 1987). However, the specificity of RsDPLA as an antagonist of bioactive LPS species and their derivatives (Kirkland *et al.*, 1991; Takayama *et al.*, 1989), coupled by its failure to inhibit TNF induced by non-LPS related compounds (Golenbock *et al.*, 1991a), suggest that RsDPLA acts by competitively inhibiting the binding of lipid A or related structures to a common receptor(s).

In this study, we first sought to assess the capacity of RsDPLA to inhibit TNF induction by four agonists: sLPS, ReLPS, DPLA, and MPL. The RsDPLA failed to induce TNF secretion at any concentration tested. In contrast, all four agonists induced TNF secretion, although sLPS was 10-fold more potent than ReLPS, 100-fold more potent than DPLA, and 1,000-fold more potent than MPL. The relative ability of RsDPLA to block TNF secretion by the four agonists was inversely proportional to their ability to induce TNF. These data are consistent with a model in which the relative cytokine inducing activity of each agonist is proportional to its relative strength of binding to LPS receptors on the cell surface. However, the gene induction data (Figure 26) suggest that binding of MPL and DPLA result in equivalent levels of TNF steady-state mRNA. Therefore, the ten-fold difference in TNF secretion exhibited by MPL *versus* DPLA may reflect post-transcriptional control. Furthermore, the findng that TNF mRNA levels induced by LPS *versus* ReLPS are equivalent suggest that signalling is greatly potentiated by additional modifications to the lipid A, i.e., that caused by the presence of the KDO or the complete polysaccharide. An inner core receptor has been demonstrated on hepatocytes (Parent, 1990) and may exist on macrophages, although this possibility has yet to be demonstrated experimentally. Thus, in this model, sLPS would bind most strongly to the lipid

A, and perhaps additional receptors, and it would be the least displaceable with RsDPLA. Conversely, MPL would bind least strongly and would be most readily excluded by RsDPLA from binding to the lipid A receptor(s). Future studies in which RsPDLA is used to block transcription induced by MPL *versus* DPLA may provide additional clues to the problem.

Significant differences in bioactivity have been attributed to the presence or absence of the position 1 phosphoryl group (Ribi, 1984). The hypothesis that removal of this phosphoryl group from the RsDPLA might result in a less effective inhibitor of LPS-induced TNF secretion, possibly due to destabilization of its interaction with LPS receptor(s) caused by loss of the large negatively charged moiety, was verified by the blocking experiments. Consistent with the finding that MPL is significantly less potent than DPLA, ReLPS, and LPS with respect to TNF secretion, RsMPLA is a less effective inhibitor than RsDPLA (Compare Figures 20 and 21) of TNF induction by those agonists which retain the position 1 phosphoryl group. In contrast, RsMPLA was almost as effective as an inhibitor of MPL as RsDPLA. These data further support the hypothesis that the interaction of LPS or LPS derivatives with the LPS receptor is strengthened by the presence of the position 1 phosphoryl group.

Macrophages have been shown to mediate the induction of "early endotoxin tolerance" (Freudenberg *et al.*, 1987). *In vitro* data presented here suggests the possibility that differential interaction of LPS or MPL with macrophages might, in part, underlie the *in vivo* observations. The use of RsDPLA to inhibit induction of "tolerance" *in vitro* has provided additional insights into the interaction of this LPS-like antagonist with macrophages. Pre-treatment of cultures with RsDPLA failed to alter macrophage responsiveness to a sLPS "challenge" 20 hours later. Thus, treatment of macrophages with

RsDPLA must not lead to a long-term receptor blockade. However, the RsDPLA was able to block the tolerance induced by sLPS and MPL. As was observed with its blocking of agonist-induced TNF secretion (Figure 20), RsDPLA was better able to reverse tolerance induced by MPL than sLPS, again suggesting that MPL interacts less avidly with the macrophage than sLPS.

Another important observation derived from these findings is that the threshold for induction of TNF secretion by LPS is ~100-fold lower than its capacity to induce *in vitro* tolerance. However, the threshold for MPL-induced TNF secretion is only 10-fold lower than the threshold for tolerance induction (Compare Figure 19 and Figure 22). This compares favorably to the observation that induction of tolerance *in vivo* by LPS occurs at concentrations ~100-fold higher than those required to induce TNF secretion, whereas for MPL, only a 10-fold difference in concentration is required to induce tolerance *versus* TNF secretion. Hence, it appears that these two agonists must deliver qualitatively different signals or that the engagement of multiple receptors differs for the two agonists. The additional observation that RsDPLA cannot effectively block TNF induced by 10 ng/ml sLPS, yet effectively blocks tolerance induced by 10-100 ng/ml sLPS (See Figures 20 and 23), suggests several additional possibilities. Tolerance induction may require a higher level of receptor occupancy than induction of TNF secretion. Or, if tolerance is due to a "receptor blockade," then the failure of RsDPLA to induce *in vitro* tolerance must relate to either a faster rate of dissociation of the bound inhibitor from the receptor, or to the agonist's (but not RsDPLA's) interaction with the receptor resulting in a decreased receptor availability, due either to physical occlusion or down-regulation of their availability. The effect of RsDPLA may be to decrease the number of available agonist binding sites.

An interesting phenomenon is seen in Figure 23 A. Blockade of TNF secretion and reversal of tolerance by *R. sphaeroides* LPS derivatives can be seen as two separate events illustrating the coordinated regulation of cytokine secretion and tolerance induction. Although tolerance induced by 100 ng/ml sLPS is reversed with RsDPLA concentrations up to 6.25 μg/ml (e.g., TNF secretion upon challenge is high), higher concentrations of inhibitor result in an apparent reinstitution of tolerance (as evidenced by decreased TNF upon "challenge"). The reinstituted suppression of TNF secretion would suggest that competition for receptor occupancy alone cannot completely account for the observed high concentration effects. Perhaps at higher concentrations of agonist and inhibitor, mixed micelles form which may increase selectively the presentation of the sLPS to the macrophage and ultimately result in a reinstitution of tolerance. Future experiments should address this possibility. Receptor interactions taken into consideration, the fact remains that significantly more MPL than LPS, ReLPS, or DPLA, is required to induce equivalent tolerance *in vivo* and this dose of MPL, nonetheless, causes the elicitation of significantly lower levels of these cytokines. Induction of lower levels of toxicity-producing cytokines may be due to differences in transmembrane signalling secondary to the differential interaction of MPL and LPS with cell surface receptors.

AOAH activity is induced by both LPS and MPL, although MPL induces somewhat less AOAH activity. The idea that MPL might be less toxic because it may be more quickly eliminated from the circulation by detoxification is, therefore, not the case. The finding that the rate of deacylation by AOAH in tolerant cells is approximately 2-3 times the rate in untreated cells suggests that AOAH may play a role in tolerance. Since the macrophage is able to detoxify LPS at a greater rate, it may sustain less physical damage from the LPS.

Experiments directed at elucidating the mechanism by which the deacylation rate increases during tolerance should establish the connection between AOAH and the pertinent biochemical pathway(s).

The LPS-inducible "early" genes first described by Tannenbaum *et al.* (1988) exhibit a complex pattern of expression. Although all six were originally cloned based on their capacity to be activated transcriptionally by LPS, their induction by other agents has since been characterized (Luster *et al.*, 1985; Ohmori *et al.*, 1990; Hamilton *et al.*, 1989; Tannenbaum *et al.*, 1989; Tannenbaum and Hamilton, 1989; Han *et al.*, 1990). Some regulatory connections among these genes have already been established. For example, the genes for IP-10, D3 and D8 have been all shown to be regulated in some fashion by IFN stimulation in macrophages and also have been found to be stimulated by platelet derived growth factor in the BALB/c 3T3 fibroblast cell line (Tannenbaum *et al.*, 1989). Transcription of gene D3 is upregulated by LPS, IFN-γ, and IFN-β. IP-10 responds best to LPS and IFN-γ (and not so strongly to IFN-β), whereas D8 responds to LPS and IFN-β, thus indicating the complex and overlapping nature of the control mechanisms involved (Hamilton *et al.*, 1989). Genes for D2 and D7 have been shown to be strongly induced by PMA, implicating the possible involvement of PKC activation in their regulation (Tannenbaum *et al.*, 1988). PKC is known to be important in enzyme activation and phophorylation control. Factors affecting the coordination of the LPS response in terms of these genes continue to be characterized.

Examined in the context of the observed disparity of *in vivo* effects caused by LPS and MPL, the similarities in the expression patterns of these genes convey new insights concerning the mode of interaction of LPS and its derivatives with macrophages. The data indicate a difference between the ability

of LPS derivatives to induce TNF mRNA and the ability to induce TNF as a secreted product (Figures 26 A and D). These results suggest that additional regulatory mechanisms, (i.e., post-transcriptional regulation) may be invoked in response to some LPS structural analogues, but not others. For example, there is good correlation between maximal TNF gene expression and maximal secreted TNF activity for both LPS and MPL in the dose response. But, for ReLPS and DPLA, results show them to be intermediate in the ability to stimulate TNF secretion, though TNF mRNA induced by ReLPS is not different from that induced by LPS, nor is the steady-state mRNA level induced by DPLA different from that stimulated by MPL. Differential response to various LPS-derivatives suggests that specific portions of the toxic LPS structure may influence secretion. For example, TNF mRNA is induced to the same steady-state level by LPS and ReLPS (Figure 26) suggesting that KDO facilitates TNF mRNA induction since these two structures are ~100-fold more efficacious than DPLA or MPL for induction of TNF mRNA. ReLPS apparently results in less efficient processing of equivalent TNF message into the secreted product, since secreted levels differ by a factor of ten-fold between LPS and ReLPS. This disparity may indicate that the loss of the inner core sugars or the O-antigen oligosaccharides contributes directly to a difference in potency at the transcriptional level for some genes. That mRNA is induced equivalently at the same concentration by DPLA and MPL for all these genes (Figure 26-28) suggests that removal of the position 1 phosphoryl group from lipid A to create the monophosphoryl derivative makes no significant difference in the interaction of these two compounds with the macrophage membrane that leads to transcription. The presence or absence of the phosphoryl group apparently makes a difference in the regulation of TNF leading to the secreted product, since in terms of secretion, DPLA is 10 times more potent than MPL. Alternately, two different receptors may be responsible

for the different effects of DPLA and MPL. This set of circumstances is extremely speculative, however, since in these experiments only one secreted product could be compared to the conditions governing the transcription of its gene. Further studies are needed to determine if this scenario is a general feature of the regulation of gene induction by each of the LPS analogues, or whether it is peculiar to TNF. We might speculate that the difference in the amount of secreted TNF is due to different efficiency of cleavage of TNF from the cell membrane initiated by various LPS derivatives, for instance.

The removal of the phosphoryl group also creates a temporal difference in the induction of TNFα and IL-1β mRNA (Figure 30). Since ReLPS and DPLA induce tolerance at the same concentration *in vivo* as LPS, but MPL does not (Figure 8), the difference in MPL's ability to induce tolerance *in vivo* may be a temporal effect resulting from a delayed transcription of factors important in the induction of tolerance, i.e., IL-1 and TNF.

IP-10 has been recently identified as a member of the platelet factor 4 family of intercrine proteins (Luster *et al.*, 1985; Ohmori and Hamilton, 1990) and its gene is structurally related to competence genes JE and KC (Narumi and Hamilton, 1991). D3 has also been tentatively identified as a member of the interferon induced 202-204 family of genes (Choubey *et al.*, 1989). The similarity of IP-10, D3, and D8 transcription patterns suggests that D8 may also be a secreted proinflammatory or chemotactic factor, which, together with IP-10, is under a separate control mechanism from the regulation of TNF and IL-1 production. These two groups of genes also differed in the degree to which their transcription of steady-state mRNA was suppressed during tolerance (Figure 34). The rapidity of downregulation of steady-state mRNA levels for D8 and IP-10 during tolerance may imply their essential nature in toxicity, but it remains to be

determined if lowered mRNA is a result of cessation of transcription, although it has been shown that the decrease in mRNA after LPS "challenge" is not due to a decrease in mRNA half-life (Virca *et al.*, 1989). It has been hypothesized that LPS-induced transcriptional "silencers" induced during tolerance (Haas *et al.*, 1990) are the reason for the decline of steady-state mRNA levels, because during tolerance, the induction of nuclear transcription factors such as NF-κB by LPS are not disrupted (Haas *et al.*, 1989; Haas *et al.*, 1990). In this regard, it would be important to examine the concurrent production of IκB in this system, since IκB has been shown to antagonize the action of NFκB (Baeuerle and Baltimore, 1988). In 70Z/3 pre-B cells, it is thought that IκB inhibitor complexes with NFkB in the cytosol, unless IκB is phosphorylated by PKC (Baeuerle and Baltimore, 1988). NFκB exists as a heterodimer formed between 50 kDa and ~65 kDa subunits, and the latter appears necessary for its binding to IκB (Collart *et al.*, 1990). When phosphorylated, IκB is no longer able to bind NFκB, and the released heterodimer translocates into the nucleus to bind promoter regions for cytokines such as TNF (Collart *et al.*, 1990), and IL-6 (Libermann and Baltimore, 1990), thus promoting transcription.

The LPS-inducible genes D2 and D7 examined in these experiments may be important factors in the induction or maintenence of tolerance, since their mRNA levels are superinduced after LPS challenge in pretreated macrophages. Perhaps these as yet unidentified genes produce transcriptional/translational inhibitory factors similar to IκB that may be required during tolerance. Alternately, D2 and D7 might be phosphorylases that are required to activate gene "silencer" activity. Since these two genes are known to be strongly induced by PMA (Tannenbaum *et al.*, 1988), perhaps activation of protein kinase C is important in the induction of tolerance to endotoxin by phosphorylation of

proteins, which either actively suppress normal gene induction, or render these products inactive as trans-acting factors. Thus, D2 and D7 gene products might also be phosphoproteins that are transcriptional regulatory factors involved in the induction of tolerance.

In fact, a specific group of proteins has been found to be phosphorylated in macrophages shortly after stimulation by LPS (Shinomiya *et al.*, 1991). LPS is incapable of stimulating the phosphorylation of at least one these proteins (~65 kDa) in *Lps*^{d} C3H/HeJ macrophages. This suggests that the LPS defect may be related to their inability to phosphorylate this protein, which is possibly a necessary transcription factor or an inhibitor analogous to IκB. It is also interesting to note that C3H/HeJ macrophages do not exhibit tyrosine phosphorylation of three other proteins (~41 - 44 kDa) that occur in normal macrophages stimulated with LPS (Weinstein *et al.*, 1991; Carl Manthey, personal communication).

Similar sets of phosphoproteins have been demonstrated to undergo activation in vascular endothelial cells which are stimulated with IL-1 (Levin and Santell, 1991). Levin and Santell showed that one particular phosphoprotein (P29) is involved in a mechanism that may be similar to that involved in tolerance induction in macrophages. This phosphoprotein was dephosphorylated over a period of time (during which the cells were hyporesponsive to repeated IL-1 treatment) by a specific, okadaic acid-inhibitable, phosphatase. The ability of P29 to be rephosphorylated coincides with return of IL-1 responsiveness. Future experiments concerning the identity of D2 and D7 gene products and the LPS-induced phosphorylation of "stress-induced" or "heat shock" proteins will be required to elucidate these many issues.

In summary, MPL is less toxic than LPS because it induces much lower levels of toxic symptom-inducing cytokines such as IL-1, TNF, IL-6 and IFN, although it induces approximately the same levels of CSF *in vivo* . This inability to produce substances which are known macrophage products may be due to MPL's relative inability to interact with macrophage LPS receptors. MPL lacks the 1 phosphoryl group, which may cause an increase in hydrophobicity, resulting in less efficient receptor contact with lower binding capacity. The resultant transmembrane signaling in macrophages is reflected in the requirement of 100-fold higher concentrations of MPL to induce known LPS-inducible genes. Of these genes, two (IL-1β and TNFα), are transcribed noticeably later by MPL, which may contribute to MPL's relative lack of toxicity as well. The comparison of LPS *versus* MPL has been useful in speculation about the functions of genes for secreted products such as IP-10, and to speculate about the possible involvement of genes D2 and D7 in the induction and maintainence of tolerance.

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